RANTES and T lymphocytes

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RANTES AND T LYMPHOCYTES

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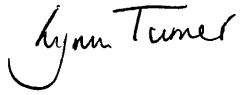

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ABSTRACT

RANTES (regulated on activation, normal T cell expressed and secreted) is a potent chemoattractant for T lymphocytes, but the signalling pathways utilised by RANTES and the other functional effects of RANTES on T lymphocytes have not been clearly defined. RANTES produced a "bell-shape-like" chemotactic response and a corresponding increase in polarisation and actin polymerisation of the human primary T lymphocytes.

In T lymphocytes loaded with fura-2 acetoxymethyl, the CD3 mAb, UCHT1, but not RANTES, elicited elevation of intracellular calcium levels. UCHT1 stimulation of T lymphocytes induced phosphatidic acid production and the breakdown of phosphatidylinositol (PtdIns) 4,5-bisphosphate but RANTES had no effect. Immunoprecipitates of PI 3-kinase, derived from T lymphocytes stimulated with RANTES, contained increased *in vitro* PI 3-kinase activity compared with that present in immunoprecipitates from vehicle-treated cells. In addition, wortmannin, a potent PI 3-kinase inhibitor, inhibited RANTES-induced T lymphocyte migration, polarisation, actin polymerisation and increased PI 3-kinase activity. Costimulation of T lymphocytes by UCHT1 and chinese hamster ovary cells expressing B7 (CHO-$B7^+$) induced RANTES peptide production. Also, when RANTES was used in addition to costimulation, it induced T lymphocyte proliferation and IL-2 receptor upregulation and above the levels seen with costimulation alone, but did not alter IL-2 production.

These results indicate that RANTES not only induces migration of T lymphocytes but plays a role in costimulation of T lymphocytes. In addition, RANTES induced migration of T lymphocytes is independent of detectable elevation but involves the PI 3-kinase signalling pathway.

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ABBREVIATIONS

Ag	antigen
Ag-MHC	antigen associated with self major histocompatibility complex
ATP	adenosine trisphosphate
$[Ca^{2+}]_i$	intracellular free calcium concentration
CHO	chinese hamster ovary cell
CPM	counts per minute
DAG	1,2 diacylglycerol
DMEM	Dulbecco's Modified Essential Medium
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme linked immunosorbent assay
ERKS	extracellular signal regulated kinases
FACS	fluorescence activated cell sorter
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
FMLP	formylmethionylleucylphenylalanine
G-actin	globular actin
G-protein	guanine nucleotide binding protein
HBSS	Hank's balance salt solution
HIBS	heat inactivated bovine serum
HIV	human immunodeficiency virus
HIV-SF	human immunodeficiency virus - suppressive factor
HPLC	high performance liquid chromatography
IP-10	interferon-inducible protein
IL	interleukin

IP_3	inositol 1,4,5-trisphosphate
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kilodalton
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
NK cells	natural killer cells
O.D.	optical density
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PI 3-kinase	phosphoinositide 3-kinase
PI 4-kinase	phosphoinositide 4-kinase
PKC	protein kinase C
PHA	phytohaemagglutinin
PLC	phospholipase C
PMA	phorbol myristate acetate
PtdIns	phosphatidylinositol
PtdOH	phosphatidic acid
RANTES	regulated on activation, normal T cell expressed and secreted
SEM	standard error of the mean
TLC	thin layer chromatography
TNF	tumour necrosis factor

TPA 12-0-tetradecanoylphorbol 13-acetate

SECTION ONE

INTRODUCTION

1.1 Background

Immune responses are dependent upon the immune system being able to recognise antigens on potential pathogens and then developing an appropriate reaction to eliminate the source of the antigen. Therefore, there are two main phases to a immune response: recognition of the antigen, which is performed by lymphocytes and the effector phase which involves many cells such as neutrophils, eosinophils, basophils, mast cells and lymphocytes.

Communication between the cells of the immune system is not only achieved through direct cell-to-cell contact, mediated by specific cell surface proteins but also through diffusible, chemical mediators called cytokines. These secreted cytokines have several advantages over direct cell-cell interaction as they can be transported quickly to sites of infection and can affect target cells far from their site of release (Miller & Krangel, 1992b).

Cytokines have several features in common. They are antigen nonspecific glycoproteins, synthesised and normally rapidly secreted in response to a stimulus. They are extremely potent mediators which interact with specific high affinity receptors on the cell surface. One cell can produce many different cytokines and these, in turn, can have multiple effects on different target cells (Oppenheim *et al.* 1991).

Many cytokine receptors require a receptor associated molecule in order to achieve high affinity ligand binding and/or transmission of cytoplasmic signals. Some of these receptor associated molecules are shared by different cytokine receptors. A common event in the cytokine system is the phosphorylation of the cytokine receptors and of various cytoplasmic proteins. Many of the cytokine receptor associated molecules are yet to be identified and how binding of cytokines to their receptors transmit intracellular signals is still very unclear (Taga & Kishimoto, 1992).

Recently, a large superfamily of structurally and functionally related cytokines has been identified and named the chemokine superfamily. It consists of a number of 5 - 10 kilodaltons (kDa) pro-inflammatory proteins that show 20 - 50 % homology at the amino acid level and display four conserved cysteine residues near the N - terminus. This superfamily has been subdivided into two subfamily branches. The C-X-C chemokine subfamily has an intervening amino acid residue between the first and second of the four conserved cysteine residues, while the C-C chemokine subfamily of proteins does not have this intervening amino acid (Oppenheim *et al.* 1991).

In the course of studying peptides released by alpha granules of platelets, haematologists were the first to encounter a member of this superfamily. Platelet factor 4 (PF4) was the first protein of this superfamily to be characterised, in 1977 (Deuel *et al.* 1977). PF4 is stored in platelet-α granules along with other proteins including two other C-X-C proteins, platelet basic protein (PBP) and its N-terminal truncation derivative, connective tissue-activating peptide III (CTAP-III). ß-thromboglobulin (ß-TG), a truncation derivative of PBP and CTAP-III, was characterised early on (Castor *et al.* 1983; Holt & Niewiarowski, 1989). Immunologists then reported the induction of gene

expression for a peptide homologous to these platelet proteins in interferon-γ (IFN-γ) stimulated macrophages and named the peptide IP-10. Unlike the C-X-C proteins stored in the platelet, IP-10 was found to be induced in cells upon stimulation, and IFN-γ is particularly potent (Luster & Ravetch, 1987).

This area of research was quiet until several related proteins were found that are strongly chemotactic for leukocytes (Baggiolini *et al.* 1989). After the discovery of interleukin-8 many research groups entered the field and soon the knowledge about C-X-C proteins increased enormously (Mazurov *et al.* 1988; Yoshimura *et al.* 1987; Walz *et al.* 1987).

The structure of the first C-C protein was deduced from the cDNA of the *LD78* gene which was cloned by subtractive hybridisation from human tonsilar lymphocytes (Obaru *et al.* 1989). Information on biological activities was obtained when two proteins from murine macrophages, macrophage inflammatory protein (MIP) 1α and 1ß, were isolated (Wolpe *et al.* 1988; Sherry *et al.* 1988). Monocyte chemotactic protein-1 (MCP-1) was isolated from several human sources, and shown to be chemotactic for monocytes, but not for neutrophils (Yoshimura *et al.* 1989; Matsushima *et al.* 1989). Other related proteins which, like MCP-1, act on mononuclear cells, I-309 and RANTES, were then isolated and characterised (Miller *et al.* 1989; Schall *et al.* 1988; Schall *et al.* 1990; Miller & Krangel, 1992a).

To date, at least ten C-C chemokines and thirteen C-X-C chemokines have been identified, either at a cDNA level or a protein level (**Table 1.1**) (Baggiolini *et al.* 1994).

Table 1.1 Human Chemokines

C-X-C Chemokines	Platelet Factor 4	PF4
	Platelet Basic Protein	PBP CTAPIII ß-TG NAP-2
	Interleukin-8	IL-8
	Interferon-Inducible Protein	IP-10
	Epithelial Cell-Derived Neutrophil-Activating Protein	ENA-78
	Granulocyte Chemotactic Protein-2	GCP-2
	GRO Proteins	GRO-α GRO-ß GRO-γ
	LPS-Induced CXC Chemokine	LIX
C-C Chemokines	Macrophage Inflammatory Proteins	MIP-1α MIP-1ß MIP-1γ
	Monocyte Chemoattractant Proteins	MCP-1 MCP-2 MCP-3
	I309	
	Regulated on Activation Normal T Cell Expressed and Secreted	RANTES
	Eotaxin	
	CFF18	
C Chemokines	Lymphotactin	

The two subfamilies can also be distinguished by their target cell specificity as the C - X - C chemokines act primarily as potent chemoattractants and activators of neutrophils although there are some exceptions. IP-10 attracts only monocytes, T lymphocytes and natural killer cells (NK cells) (Yatani *et al.* 1987; Kaplan *et al.* 1987). C-C chemokines attract monocytes, T lymphocytes, basophils and eosinophils (Baggiolini *et al.* 1994). This specific targeting of leukocyte populations may be of potential therapeutic interest.

The chromosomal location of the chemokine genes is also another way of subdividing the superfamily. C-X-C chemokine genes are localised on chromosome 4 (q12 - q21) and the C-C chemokine genes at chromosome 17 (q11 - q21) (Donlon *et al.* 1990).

In the last year, a protein that is a chemoattractant for T lymphocytes has been identified and named lymphotactin. Lymphotactin has sequence homology with both subfamilies of the chemokine superfamily and only has two of the four conserved cysteine residues. The chromosomal location is also different with the gene for human lymphotactin being located on human chromosome 1. This suggests the existence of a third subfamily of chemokines (Kelner *et al.* 1994; Kennedy *et al.* 1995).

1.2 RANTES

1.2.1 Gene and Protein Structure

The discovery of RANTES occurred during the search for genes that had interesting expression properties, genes that were only expressed in T lymphocytes. In 1988, *Schall et al*, using a subtractive hybridisation method, produced a T lymphocyte minus B lymphocyte cDNA library. This library was then screened with a radioactive cDNA probe which had been prepared by subtracting cDNA from a growth factor dependent T lymphocyte cell line after antigen stimulation from B lymphoblastoid cDNA. This produced genes that would only be present after activation of T lymphocytes. A gene was identified using this method and was named *RANTES*, regulated on activation, normal T cell expressed and secreted (Schall *et al.* 1988).

In situ hybridisation experiments and analysis of somatic cell hybrids with a cDNA probe to the gene were performed. The gene is localised to the human chromosome 17 (q 11.2 - 12), as are all known C-C chemokine genes (Donlon *et al.* 1990). The RANTES gene spans approximately 7.1 kilobases (kb) which makes it the largest C-C chemokine gene identified. It consists of three exons of 133, 112 and 1075 bases, and two introns of 1.4 and 4.4 kb. The first exon contains the 5' untranslated region and the nucleotides that code for the signal sequence, the second exon encodes for the amino-terminal end of the mature protein and the third exon, the carboxyl-terminal and the 3' untranslated region (Nelson *et al.* 1993).

Over twenty potential binding sites for transcription factors were detected in the immediate upstream region of the RANTES gene e.g. consensus sites for AP-1, NFκB,

CD28RE and NFIL6 (Nelson *et al.* 1993; Ortiz *et al.* 1996). These regulatory sites were originally described in promoters from various cells not just T lymphocytes, including fibroblasts and some are known to be sites for factors that are responsive to different second messenger stimulation such as an AP-1 binding site (Lamb & MvKnight, 1991). The presence of these sites does not necessarily mean that they are important in RANTES expression but it does suggest that there will be many transcriptional controls.

The cDNA encodes a polypeptide of 91 amino acids which has no potential sites for N - linked glycosylation and includes an amino-terminal signal sequence of 23 amino acids. After cleavage of the signal sequence the mature secreted protein consists of 68 amino acids. The peptide is basic with an isoelectric point (pI) of 9.5 (Schall *et al.* 1988).

The RANTES peptide was expressed in the human embryonic kidney cell line 293. The peptide is a recombinant 8 kDa molecule, which is consistent with the predicted size of the peptide after the signal sequence has been removed (Schall *et al.* 1990). To date, the three dimensional structure of only five human chemokines has been published including RANTES. The basic monomer structure of each chemokine is similar consisting of a three stranded anti-parallel ß-pleated sheet overlayed by a carboxy terminal α helix. The conserved cysteine residues are joined by two disulphide bridges in a 1-3 and 2-4 motif (Chung *et al.* 1995).

However, the aggregation state and oligomeric structures observed by NMR spectroscopy and X-ray are different. Initially the three dimensional structure for RANTES was generated assuming that it would be a monomer at the concentrations

used, but the spectra produced proved that this was an incorrect assumption. There is also evidence from gel filtration experiments that RANTES exists as a dimer in solution at higher concentrations (Paolini *et al.* 1994). The RANTES dimer forms an elongated prolate ellipsoid with the two monomers being superimposable (Chung *et al.* 1995). This is very similar to the three dimensional structure of MIP-1ß but not similar to the compact structure of the C-X-C chemokines such as IL-8 (Lodi *et al.* 1994; Clore & Gronenborn, 1995). It is interesting that the dimer arrangements of all the chemokines appears to subdivided the superfamily into its two branches. However, it is uncertain whether the chemokines act as monomers or dimers at their receptors so the three dimensional structure differences may not be responsible for the cell-type specificity of the two subfamilies. It is more likely due to receptor-ligand contacts.

1.2.2 Cellular Sources of RANTES

Northern blot analysis has been used to identify RANTES gene expression. The RANTES mRNA was first shown to be expressed in several growth factor (interleukin-2) dependent T lymphocyte cell lines but not in all T lymphocyte or B lymphocyte tumour cell lines. Stimulation of one T lymphocyte cell line actually leads to a reduction in RANTES expression (Schall *et al.* 1988).

RANTES mRNA expression in peripheral blood mononuclear cells (PBMC) is detectable at low levels but these levels increase after antigen stimulation or phytohaemagglutinin (PHA) stimulation, after about five days incubation (Schall *et al.* 1988). However, another group could detect RANTES expression in resting PBMC easily but were unable to detect changes after antigen stimulation even after seven days

(Miller *et al.* 1989). These conflicting results could be due to differences in methods for preparation of the PBMC, as RANTES expression could possibly be upregulated during the preparation stages.

In the last four years, studies have shown that T lymphocytes are not the only source of RANTES. A wide range of cell types express RANTES including natural killer cells, renal mesangial and epithelial cells, human lymph nodes, tumours, fibroblasts, and vascular smooth muscle cells (**Table 1.2**).

Table 1.2 RANTES mRNA Tissue Expression

Cell Source	Stimuli	References
T Lymphocytes	Constitutive Phytohaemagglutinin (PHA) Anti-CD3	(Schall *et al.* 1988; Miller *et al.* 1989)
Platelets	Constitutive	(Kameyoshi *et al.* 1992)
Fibroblasts	Tumour Necrosis Factor-α(TNF-α)	(Jordan *et al.* 1996a)
Natural Killer Cells	Constitutive	(Nelson *et al.* 1993)
Renal Epithelial and Mesangial Cells	TNF-α/IL-1ß	(Wolf *et al.* 1992)
Human Lymph Nodes with DTH Lesions	Constitutive	(Devergne *et al.* 1994)
Solid Tumours	Constitutive	(Schall, 1991)
Osteoblast Cells	Constitutive	(Schall, 1991)
Myeloid Precursor Cells	Constitutive DMSO	(Schall, 1991)
Vascular Smooth Muscle Cells	TNF-α	(Jordan *et al.* 1996b)

1.2.3 Receptor - Characterisation

The first step in chemokine activation of inflammatory cells involves binding of the chemokine to specific cell surface receptors. The increasing availability of purified recombinant chemokines has made the identification and molecular cloning of chemokine receptors possible. In the last four years five different C-C chemokine receptors have been identified.

cDNA has been isolated, from haematopoietic cells, that encodes a receptor for RANTES which binds MIP-1α, RANTES, MCP-1 and MCP-3 with varying affinities (Gao *et al.* 1993; Neote *et al.* 1993; Combadiere *et al.* 1995b). It conforms to the classical seven transmembrane spanning domain architecture of a G protein coupled receptor. This is also consistent with the fact that many functions of the C-C chemokines can be blocked by pertussis toxin. Transcripts for this MIP-1α/RANTES receptor have been detected in HL60, U937, THP-1 cell lines and human B lymphocytes but not detected in total RNA from activated human T lymphocytes or T cell lines. Also, using binding studies and functional assays, the MIP-1α/RANTES receptor has been identified on monocytes and on a differentiated HL60 cell line (Van Riper *et al.* 1993; Wang *et al.* 1993; Van Riper *et al.* 1994). It is now generally accepted that all these studies are referring to one G protein coupled receptor known as C-C CKR1, which binds MIP-1α, MCP-1 and RANTES. Although the binding affinities for the chemokines differ depending on the cells examined.

A C-C CKR2 has also been identified on THP-1 cells and MonoMac6, another monocytic cell line, which binds only MCP-1 and MCP-3. There are several variants of this receptor as it can be alternatively spliced at the C-terminal end (Charo *et al.*

1994). C-C CKR3 is an eosinophilic receptor which binds eotaxin, RANTES and MIP-1α (Combadiere *et al.* 1995a). The most recent discovery is C-C CKR4, found on a human basophilic cell line, KU812. This receptor can be activated by MCP-1, MIP-1α and RANTES (Power *et al.* 1995).

An ubiquitous chemokine receptor has been found on red blood cells and endothelial cells and lymphocytes, known as Duffy antigen (Kunkel *et al.* 1995). This is a multispecific chemokine receptor which binds chemokines from the C-X-C subfamily such as IL-8 and chemokines from the C-C subfamily including RANTES and MCP-1 (Zhao-hai *et al.* 1995). This appears to be the only chemokine receptor to be nonspecific for the two subfamilies. There is no evidence to suggest that the receptor is regulated by G proteins. The Duffy antigen, which is a receptor for the human malarial parasite *Plasmodium vivax* has not been shown to transduce signals and is thought to promote clearance of chemokines from the circulation (Horuk, 1994; Horuk *et al.* 1994).

1.2.4 Biological Activities of RANTES

1.2.4.1 Mononuclear Cells

Recombinant RANTES has been used to assess the biological effects of this chemokine. RANTES caused the selective migration *in vitro* of a subset of T lymphocytes which expressed the cell surface antigens , CD4 and CD45RO$^+$ (Schall *et al.* 1990)(**Table 1.3**). This specific isoform of CD45 is thought to be present on prestimulated or primed memory T_H lymphocytes. These are immunologically experienced cells with

enhanced proliferative and cytokine producing potentials. RANTES can also elicit transendothelial chemotaxis of unstimulated primed memory T lymphocytes in a chemotaxis assay using human umbilical vein endothelial cell monolayers (Roth *et al.* 1995).

A human/severe combined immune deficient (SCID) mouse model was used to demonstrate infiltration of human T lymphocyte *in vivo* in response to RANTES. SCID mice received human lymphocytes followed by sequential injections of RANTES. Seventy two hours after injection of RANTES, both $CD4^+$ and $CD8^+$ migrated into the skin in response to RANTES (Murphy *et al.* 1994).

RANTES also acts as a chemoattractant for human blood monocytes and THP - 1 cells *in vitro* (Wand *et al.* 1993; Schall *et al.* 1990). The first studies performed looked at the effects of RANTES, in solution, on monocyte migration, which is known as chemotaxis. More recently, the haptotaxis of monocytes was shown to be induced by RANTES *in vitro* (Wiedermann *et al.* 1993). Haptotaxis is defined as cell migration induced by surface bound chemotactic gradients. This suggests that RANTES may not just induce the migration of inflammatory cells in inflamed tissues, but may also cause these cells within the bloodstream to adhere to the endothelium and extravasate into the tissues. RANTES can also trigger the release of N-acetyl-ß-D-glucosaminidase, a granular enzyme, from monocytes that have been treated with cytochalaisin B, to prevent them adhering (Uguccioni *et al.* 1995).

From all this information a model can be proposed to explain the role of chemokines such as RANTES and mononuclear cells in inflammation. During the first phases of

the inflammatory response, the endothelium is activated and expresses adhesion molecules of the selectin family. These molecules cause leukocytes to roll along the vascular wall. Platelets can also degranulate, releasing RANTES which can bind to the activated endothelium and provide a haptotactic gradient for a specific subsets of mononuclear cells. As the inflammatory process continues, IL-1ß and TNF-α are produced, upregulating the production of chemoattractants such as IL-8, MIP-1α and RANTES which provide more of a chemotactic/haptotactic gradient into the interstitium. In combination with the induced expression of specific adhesion molecules of the integrin and immunoglobulin superfamilies, these chemokines lead to mononuclear cell extravasation. The final stage occurs, after several days, when T lymphocytes attracted to the inflammatory site become activated and strongly express RANTES as they become functionally active. This helps to amplify the immune response as more memory T lymphocytes are recruited to the site of inflammation (**Fig. 1.1**).

1.2.4.2 Granulocytes

Neutrophil migration is not induced by RANTES (Schall *et al.* 1990). However, eosinophils are another inflammatory cell which can be activated by RANTES (Kameyoshi *et al.* 1992). RANTES is also a powerful chemoattractant of eosinophils *in vitro*, as effective as C5a (Rot *et al.* 1992). C5a is a component of the complement cascade system, part of the immune response, and a potent eosinophil and neutrophil chemoattractant. However, some groups have shown that eosinophils have a requirement for priming before migrating in response to RANTES (Schweizer *et al.* 1994). This may be due to differences in chemotactic methods used by the different

groups or by variations in blood donors, some groups having used allergic subjects. RANTES also induces production of reactive oxygen species (an indicator of the release of toxic granule proteins after degranulation) from cytochalaisin-B treated cells but it is a weaker inducer than C5a (Kapp *et al.* 1994). Eosinophil transendothelial migration is also induced by RANTES *in vitro* and can be inhibited by antibodies against the ß2 integrin CD18 adhesion complex. RANTES did not cause an increase in adhesion molecule expression or adhesion of eosinophils to endothelial cells (Ebisawa *et al.* 1994).

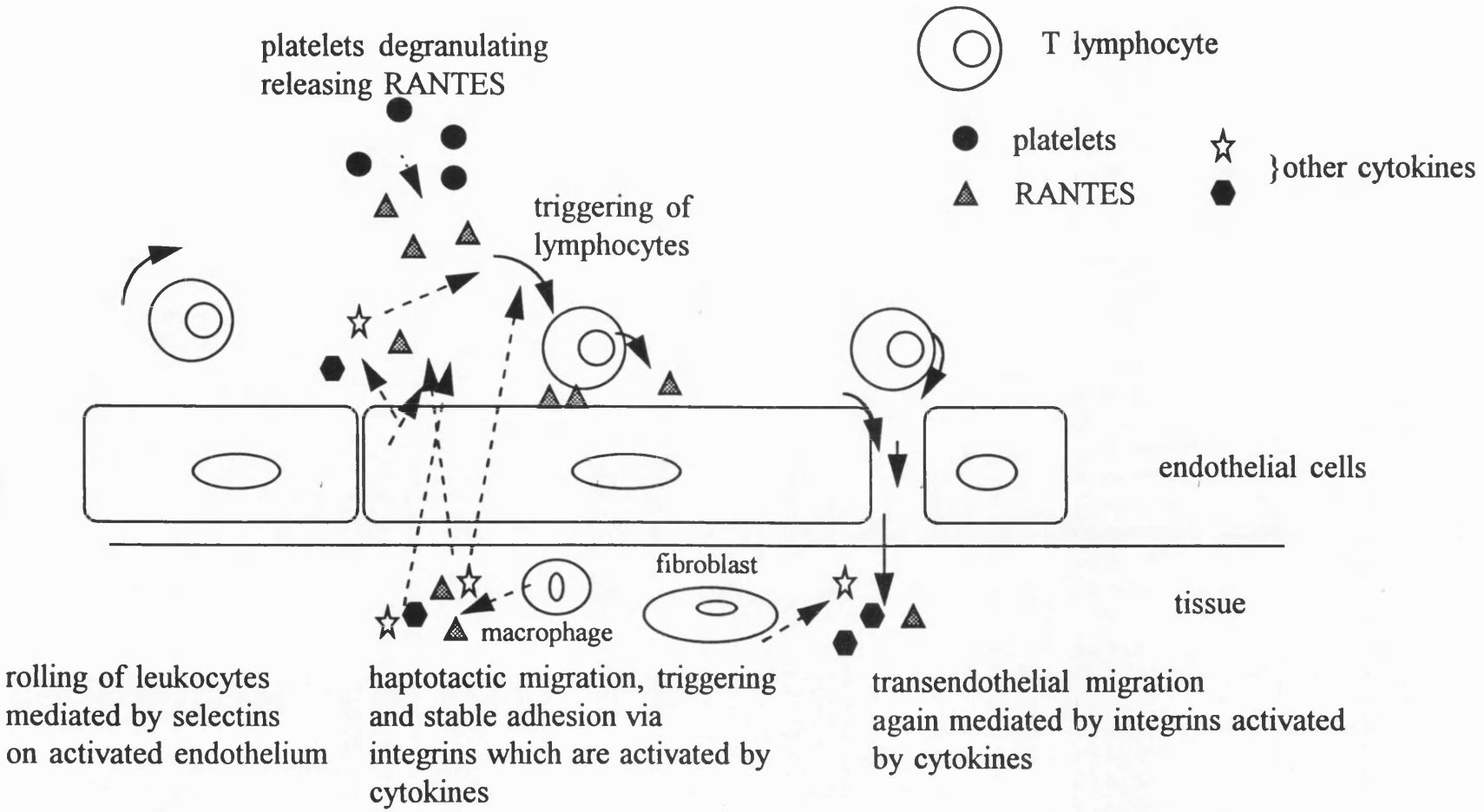


Fig. 1.1 Model of Lymphocyte Transendothelial Migration

Basophils have also been stimulated with RANTES. The chemokine is an effective basophil chemoattractant but a weak stimulator of mediator release such as histamine and leukotrienes from these granulocytes (Kuna *et al.* 1993). These effects of RANTES on granulocytes are not necessarily always beneficial. The degranulation of eosinophils is responsible for some of the pathologic changes observed in allergic diseases of the airways or skin where the immune responses are inappropriate to the antigen challenge (Charlesworth *et al.* 1989; Schweizer *et al.* 1994). RANTES can also be detected in lymph nodes of allergic individuals (Devergne *et al.* 1994).

1.2.4.3 Tumours

RANTES also induces the chemotaxis *in vitro* of the anti-tumour cells, NK cells and IL-2-activated NK cells (IANK cells) (Maghazachi *et al.* 1994). These cells have high anti-tumour activity and have been used to treat murine metastases but do not disperse efficiently to the site of tumour growth. Therefore RANTES could be used to enhance the accumulation of these cells at tumour sites. This therapeutic potential for RANTES is also supported by the knowledge that RANTES production has been detected in solid tumours, suggesting it has a role in causing the selective migration of inflammatory cells with certain tumours (Schall, 1991).

1.2.4.4 Human Immunodeficiency Virus

Evidence has suggested that $CD8^+$ T lymphocytes have some function in the control of human immunodeficiency virus (HIV) infection *in vivo*, either by cell mediated mechanisms or by the production of HIV-suppressive factors (HIV-SF). Recently,

Cocchi et al have demonstrated that several chemokines including RANTES can mediate antiviral effects and identified RANTES, MIP-1α and MIP-1ß as the major HIV-SF, produced by $CD8^+$ T lymphocytes (Cocchi *et al.* 1995).

Summary

The specificity of RANTES does make it an attractive candidate for roles in conditions where T lymphocytes, eosinophils and basophils predominate, including auto-immune disease such as rheumatoid arthritis. RANTES may also play a part in T lymphocyte memory function due to its specific effects on memory T lymphocytes and have a role in potential therapies for the prevention and therapy of AIDS.

Table 1.3 Biological Activities of RANTES.

Cell Target	Effects of RANTES	References
Memory T Lymphocytes	Chemotactic	(Schall *et al.* 1990)
Monocytes	Chemotactic Haptotactic Calcium Fluxes Release of Granular Enzymes	(Wang *et al.* 1993; Schall *et al.* 1990; Wiedermann *et al.* 1993; Uguccioni *et al.* 1995)
Eosinophils	Chemotactic Transendothelial Migration Release of Toxic Granule Proteins	(Rot *et al.* 1992; Schweizer *et al.* 1994; Kim *et al.* 1994; Ebisawa *et al.* 1994)
Basophils	Chemotactic Weak Mediator Release	(Kuna *et al.* 1993)
Natural Killer Cells	Chemotactic	(Maghazachi *et al.* 1994)

1.3 Signalling Pathways

To date, three signalling pathways have been shown to be important in T lymphocyte activation : i) protein tyrosine kinases (PTK), ii) phospholipase C (PLC), and iii) phosphoinositide 3-kinase.

The first pathway involves the binding of extracellular ligands to receptors that interact with PTK from the *src* (e.g. Lck, Fyn) and *syk* (e.g. Syk and ZAP70) families, causing an increase in tyrosine protein phosphorylation. Coupling of the T lymphocyte antigen receptor (TCR) to cytoplasmic tyrosine protein kinases is a property of the cytoplasmic domain of the receptor. Chimeric proteins containing the cytoplasmic domain of the CD3-zeta chain can stimulate tyrosine phosphorylation, inositol phosphate metabolism, calcium mobilisation and IL-2 production (Irving & Weiss, 1991). Cytoplasmic domains of other TCR chains have also been shown to stimulate tyrosine protein kinases (Letourneur & Klausner, 1992).

The cytoplasmic domains all share a sequence motif, called a TAM (tyrosine-based activation motif)(sometimes referred to as the antigen-recognition activation motif, ARAM)(Samelson & Klausner, 1992). In resting T lymphocytes, this motif associates with the amino-terminus of *syk* kinases (Timson Gauen & Kong, 1992). Receptor ligation induces receptor tyrosine phosphorylation and tyrosine residues in the TAM motif are phosphorylated. This probably explains the subsequent binding of *syk* kinases via their src-homology (SH)2 domains to the motif (Sancho *et al*, 1993 ; Pawson & Gish, 1992), which initiates a signalling cascade resulting in lymphocyte activation.

The prior phosphorylation of the TAM may be fulfilled by the *src* kinases. COS cells

transfected with T cell receptor chains (CD8-zeta) have been transiently transfected with cDNAs encoding for various kinases. Phosphorylation of the receptor chains and *syk* kinase recruitment was dependent upon coexpression of *lyk* or *fyn* (Chan *et al*, 1992; Iwashima *et al*, 1992). In addition, T lymphocyte activation has been shown to be dependent upon *lck* by using non-responsive T lymphocyte cell lines that have no endogenous *lck*. When *lck* is introduced into these cells, the cells can then respond to signalling through the antigen receptor (Strauss & Weiss, 1992).

The second pathway, the classical phosphoinositide pathway, is activated when agonists bind to G protein or PTK coupled receptors, causing PLC activation and the production of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol.

The third pathway also involves phosphatidylinositol (PtdIns) signalling, but this time the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) is activated and the second messengers are 3-phosphorylated inositol lipids (D-3 phosphoinositides). Only the two pathways involving PtdIns signalling were studied.

1.3.1 Classical Phosphoinositide Pathway

Initially, the investigations into PtdIns signalling, in this study, were centred around the G protein coupled PLC and its substrate phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$), as the receptors identified for RANTES have all conformed to the classical seven transmembrane spanning domain architecture of G protein coupled receptors (see 1.2.3). This is referred to as the classical phosphoinositide pathway

(Berridge & Irvine, 1984). PtdIns is an acidic phospholipid mainly located in the inner plasma membrane. PtdIns(4,5)P_2 is formed by the phosphorylation of PtdIns by specific kinases. The binding of certain agonists to G protein coupled cell surface receptors is followed by activation of a heterotrimeric G protein. An exchange occurs in the α subunit of the G protein from a GDP- to a GTP- bound state, resulting in a dissociation of the α subunit from the $\beta\gamma$ subunits. The free α subunit can activate both PLC ß1 and ß2, whereas the free $\beta\gamma$ complex activates preferentially the PLC ß2. The activated PLCs hydrolyse the phosphodiester bond linking the phosphatidylinositol unit to the acylated glycerol moiety. This results in the production of two biological second messengers : IP_3 and 1,2 - diacylglycerol (**Fig. 1.2**).

IP_3 is a short lived messenger, lasting only a few seconds. Most cells express several of the five isoforms of the IP_3 receptor. The human type 1 IP_3 receptor has been detected on T lymphocytes (Harnick *et al.* 1995). Activation of the IP_3 receptor causes the rapid release of Ca^{2+} from intracellular stores, such as the endoplasmic reticulum, which then may cause the observed calcium influxes from the extracellular environment, referred to as capacitative calcium entry, by the release of a calcium influx factor (Berridge, 1984; Berridge & Irvine, 1984; Berridge, 1993; Putney & Bird, 1993). The influx of Ca^{2+} is responsible for a sustained phase of Ca^{2+} elevation and the replenishment of intracellular stores. Alterations in intracellular calcium levels are observed after the engagement of the TCR. Diacylglycerol release is more prolonged and it serves as a messenger by activating protein kinase C (PKC), while remaining bound to the membrane (Nishizuka, 1984; Nishizuka, 1988). This enzyme then phosphorylates serine and threonine residues in numerous target proteins. PKC can activate several signalling cascades such as ERKS and JNK which are known to regulate

transcription factors such as AP-1, which controls expression of various cytokine genes, such as IL-2 (Whitman & Cantley, 1988).

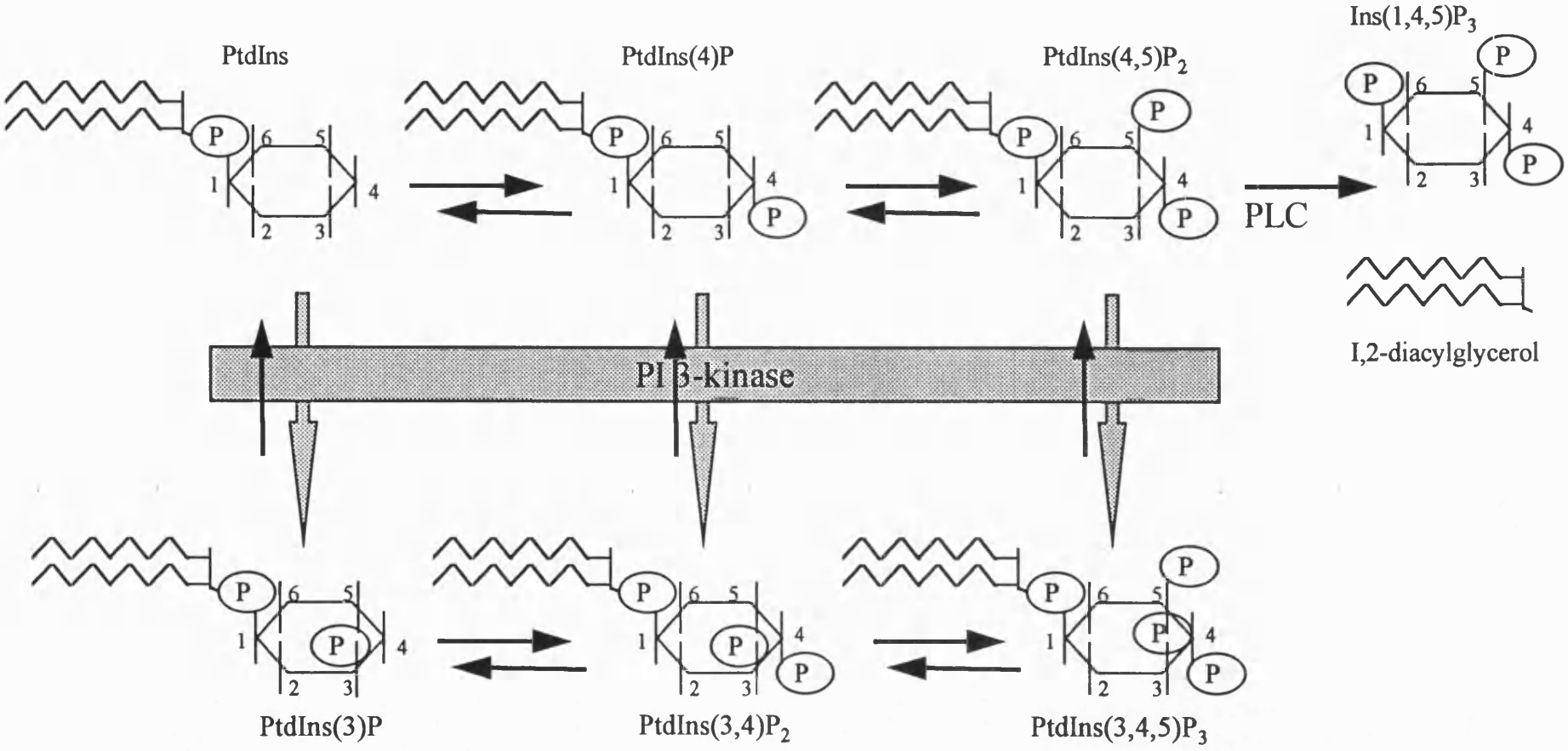


Fig. 1.2 Phosphoinositide Signalling Pathways

1.3.2 PI 3-Kinase

More recently, investigations have been based around the other PtdIns signalling pathway which involves D-3 phosphoinositides, a family of membrane phospholipids which are phosphorylated at the 3-position of the inositol ring (Stephens *et al.* 1993)(**Fig. 1.2**). They were originally discovered in transformed cells but are also found in cells such as neutrophils, platelets, brain cells and T lymphocytes (Whitman *et al.* 1988). Only very small amounts of D-3 phosphoinositides are present in cells, i.e. < 0.1 % total PtdIns lipids. The family includes phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). The routes of metabolism of these D-3 phosphoinositides are only poorly understood when compared with those of the classical phosphoinositides. No phospholipase has been found that can hydrolyse these 3-phosphoinositides (Stephens *et al.* 1993). This suggests that it is the D-3 phosphoinositides that are the signalling molecules and not any breakdown products as in the case of the classical phosphoinositides.

1.3.2.1 PI 3-Kinase Isoforms

D-3 phosphoinositides are the *in vitro* products of a growing family of enzymes referred to as PI 3-kinases (Fry, 1994) :

i) PTK/SH2 linked isoform : a heterodimer of a 85 kDa regulatory subunit (p85α,ß) (Otsu *et al.* 1991) and a 110 kDa catalytic subunit (p110α,ß) (Hiles *et al.* 1992; Fry, 1994). The p85 subunit possesses two Src homology 2 (SH2) domains, one SH3 domain, and two proline rich regions (Otsu *et al.* 1991). These may facilitate numerous

protein-protein interactions. Consensus binding motifs for the SH2 domains, YXXM, are found in the intracellular domains of receptor tyrosine kinases such as the PDGF ß - receptor; at several positions in intracellular substrates such as insulin receptor substrate - 1(IRS-1); in cytosolic receptor linked protein tyrosine kinases such as c-Src; and in cell surface receptors such as CD28 (see 1.4). Binding of Src-family kinases (such as Lyn and Fyn) via SH3 domains to the proline rich regions on p85 has also been proposed to activate PI 3-kinase via receptor tyrosine kinases (Pleiman *et al.* 1994). This isoform can use PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as its substrates *in vitro*.

ii) PI 3-kinaseγ isoform : is G protein ßγ subunit responsive and has been detected in human neutrophils, U937 cells and platelets (Stephens *et al.* 1994; Thomason *et al.* 1994). This also explains pertussis toxin inhibiting G protein receptor coupled PI 3-kinase activity as G ßγ is derived from the pertussis toxin sensitive G_i and/or G_o (Stephens *et al.* 1994). The molecular structure of the G protein sensitive PI 3-kinase has not been defined. The previously identified p85/p110 PI 3-kinase heterodimer could be activated by G ßγ as well as by phosphotyrosine. Or there may be another distinct isoform of PI 3-kinase (Stephens *et al.* 1994). The first option seems more likely as formylmethionylleucylphenylalanine peptide (FMLP) activation of neutrophils can be completely inhibited by both pertussis toxin and wortmannin (Thelen *et al.* 1994). This isoform can use PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as its substrates *in vitro*.

iii) PtdIns-specific isoform : a heterodimer of a 110 kDa and 150 kDa subunit *in vivo*. This isoform has a substrate specificity for PtdIns only and is the mammalian homolog of a yeast enzyme, Vps34, which also only phosphorylates PtdIns (Volinia *et al.* 1995).

Unless otherwise stated PI 3-kinase refers to the PTK/SH2 linked isoform.

1.3.2.2 Downstream Targets

The downstream targets of the D-3 phosphoinositides are still unclear. The homology of PtdIns-specific PI 3-kinase isoform with Vps34, which is involved in vacuolar processing and protein trafficking, suggests that PtdIns-specific PI 3-kinase and PI(3)P in mammalian cells could be involved in vesicular trafficking. The role of PtdIns(3,4)P_2 is unknown. It may be a breakdown product in certain systems but in other systems a PtdIns(3)P 4 - kinase has been described suggesting that PtdIns(3,4)P_2 may have a functional role (Graziani *et al.* 1992).

An important tool in the clarification of PtdIns(3,4,5)P_3 function is wortmannin. Wortmannin is a fungal metabolite which is cell permeant. It has been demonstrated to be a potent inhibitor of PI 3-kinase (Yano *et al.* 1993; Woscholski *et al.* 1994) and is a non-competitive inhibitor with respect to ATP or PtdIns and binds irreversibly to the p110 catalytic subunit (Arcaro & Wymann, 1993). Protein kinases A, C and G, phosphatidylinositol 4-kinase and Vps34 have also been reported not to be inhibited by wortmannin at concentrations of up to 1 μM (Arcaro & Wymann, 1993; Yano *et al.* 1993). Although a soluble form of PI 4-kinase has recently been shown to be inhibited by wortmannin at low concentrations (Nakanishi *et al.* 1995), and *in vitro* PLA_2 can be

inhibited partially by wortmannin (Cross *et al.* 1995), it is still considered as a specific PI 3-kinase inhibitor. In addition, there is one report which shows that wortmannin inhibits receptor - mediated activation of phospholipase D (PLD). The maximum inhibition of PLC, at concentrations as high as 1 μM, is 50 %. Also, this PLD inhibition could be accounted for by an indirect effect with PI 3-kinase acting upstream of PLD (Gelas *et al.* 1992) (**Table 1.4**).

Table 1.4 Summary of Concentration of Wortmannin Required to Inhibit Enzymes

Kinase	Wortmannin IC_{50} (μM)
PTK/SH2-coupled PI 3-kinase	< 0.01
Mammalian PtdIns 4-kinase - membrane bound - soluble	 > 1 0.05
PLA_2	0.002
PKC	~ 10
PLD	> 0.01

Wortmannin has been used to great effect to identify the processes in which D-3 phosphoinositides are involved and any potential downstream targets. There are at least three main downstream target molecules particularly relevant to T lymphocyte activation:

i) Protein kinase C isoenzymes

D-3 phosphoinositides can activate PKC isoenzymes such as PKCδ, ϵ , ζ and η (Nakanishi *et al.* 1993; Toker *et al.* 1994). PKC regulates many signalling pathways,

including those mediated by Ras, mitogen-activated protein (MAP) kinases such as extracellular signal regulated kinases (ERKS) and c-Jun N-terminal kinase (JNK)(Pastor *et al.* 1995). There is also circumstantial evidence to suggest that PKC isoforms could mediate mitogenic effects of the PI 3-kinase pathway, as PKCζ has been reported to be critical for mitogenic signal transduction in fibroblasts and a dominant negative PKCζ subspecies blocked NF-κB activation (Berra *et al.* 1993; Diaz-Meco *et al.* 1993).

ii) GTP-binding proteins

The Ras GTPases are a superfamily of small GTP-binding proteins which have been subjected to intensive study because activated mutants of these genes have been found in a large number of human tumours. A subgroup of this superfamily are the Rho GTPases - Rac1 and Rac2, Rho A, B, C and G, Cdc42, and TC10 (Boguski & McCormick, 1993). They function as molecular switches, and are on in the GTP-bound state, off in the GDP-bound state.

Ras stimulates both the Rac and ERKS but Rac is also activated by receptor tyrosine kinases through PI 3-kinase although the exact signalling pathways are still unclear (Hu *et a.l* 1995). Rac controls several signalling pathways including the organisation of the actin cytoskeleton by induction of lamellipodia and membrane ruffling (Zigmond 1996), activation of p21-associated kinases (PAK)(Manser *et al.* 1994), activation of the JNK pathway and stimulation of arachidonic acid metabolism, which leads to Rho activation (Macara *et al.* 1996). Rho activation can then lead to activation of PI 3-kinase (Zhang *et al.* 1993), activation of PI 5-kinase (Chong *et al.* 1994), activation of a putative MAP kinase module and phospholipase D activation (Macara *et al.* 1996). The different signalling pathways activated by the various Rho

GTPases are integrated at the level of transcription factors.

Constitutively active Rho is weakly transforming in NIH 3T3 fibroblasts and Rac and Rho have also been shown to be essential for transformation by Ras, which has suggested a role for Rho family members in controlling cell proliferation. The signalling pathways used by Rac and Rho to control cell proliferation still remain to be identified. Many of the enzymes activated downstream of Rho including PI 3-kinase and PI 5-kinase are thought to have a role in growth control (Zhang J. *et al*, 1993; Chong L.D. *et al*, 1994).

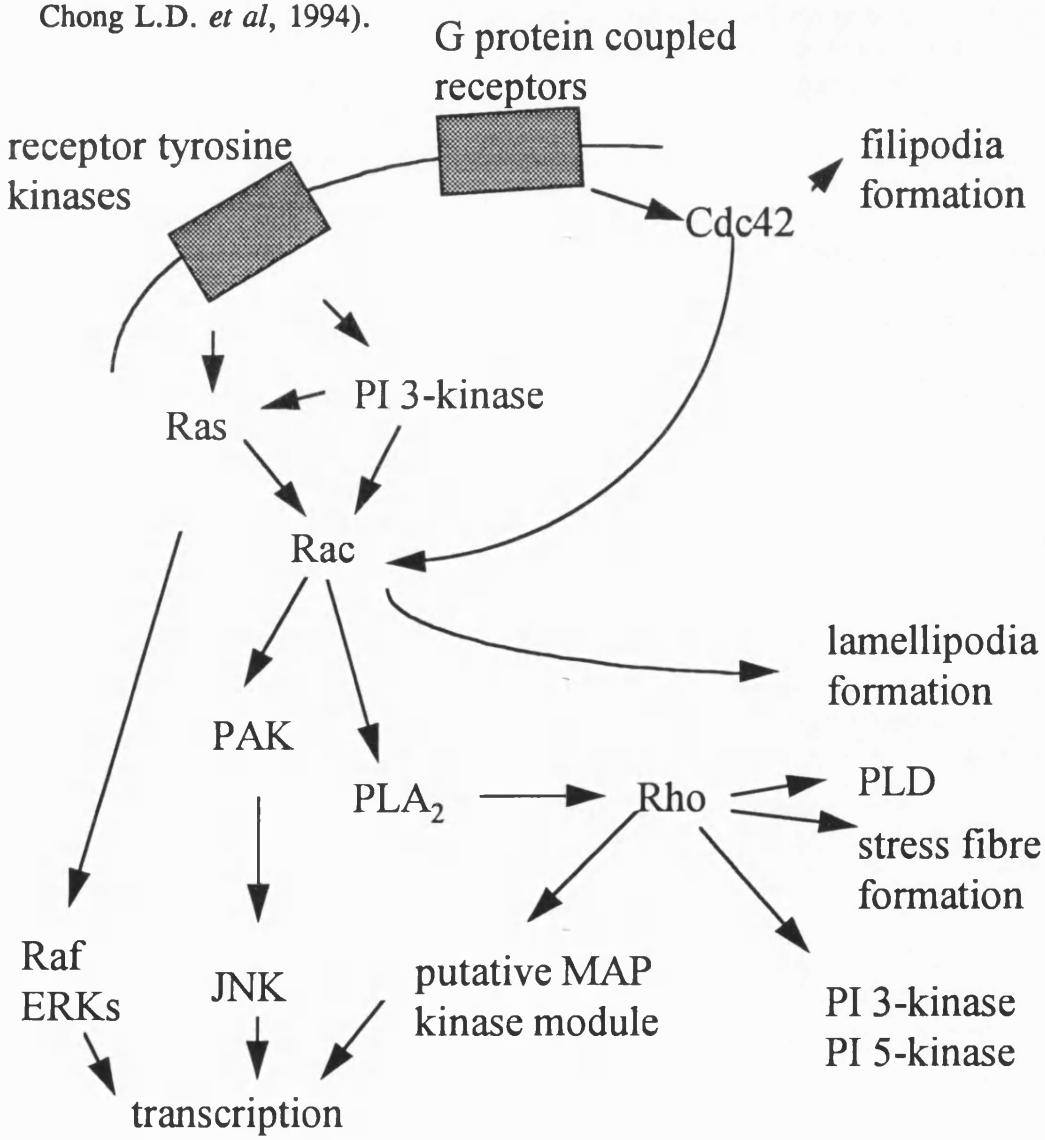


Fig. 1.3 Rho family GTPases

iii) $p70^{S6K}$

The S6 ribosomal protein , which is necessary for cells to enter the S-phase of the cell cycle after mitogen stimulation, is phosphorylated by p70 S6 kinase ($p70^{S6K}$) (a serine/threonine kinase)(Kozma & Thomas 1994). Initially $p70^{S6K}$ *in vitro* was shown to be inhibited unless phosphatase inhibitors were present and treatment with protein phosphatase-2A led to inactivation, suggesting that phosphorylation by a serine/threonine kinase was involved in regulating $p70^{S6K}$ activity (Ballou *et al.* 1988). Recently Kozma *et al* have shown that phosphorylation of Ser/Thre residues does accompany $p70^{S6K}$ activation (Kozma *et al*, 1993).

An important tool in the identification of upstream regulation of $p70^{S6K}$ is rapamycin. This inhibits T lymphocyte activation by blocking the G1 phase of the cell cycle (Kunz & Hall 1993) and selectively prevents the activation of $p70^{S6K}$ (Terada *et al* , 1993). Rapamycin does not inhibit the MAP kinase cascade. Rapamycin causes the dephosphorylation of $p70^{S6K}$ at sites that are phosphorylated in response to stimuli that activate $p70^{S6K}$ (Han *et al*, 1995). Interestingly, wortmannin and SQ20006 (a methylxanthine, inhibitor of cAMP-phosphodiesterase) also cause the dephosphorylation of these sites, although the action of this third inhibitor does not seem to be related to increased levels of cAMP and may be as a result of the inhibition of a protein kinase upstream. Rapamycin and SQ20006 both inhibit the activation of $p70^{S6K}$ by phorbol esters, whereas wortmannin does not (Han *et al*, 1995).

The mechanism of action of rapamycin is still unclear, rapamycin binds to a protein known as FKBP12 (FK506-binding protein 12 (FK506 is another immunosuppressant)) and it is the resulting complex that interacts with the target protein, mTOR (also called

FRAP)(Sabatini *et al*, 1995). Rapamycin blocks the autophosphorylation of mTOR, suggesting that the kinase activity of mTOR is necessary for its role in activating $p70^{S6K}$ (Brown *et al*, 1995). However, wortmannin does not inhibit this autophosphorylation of mTOR. mTOR has not been shown to phosphorylate $p70^{S6K}$ but as rapamycin decreases the phosphorylation of $p70^{S6K}$, a protein kinase or phosphatase is likely to be involved between mTOR and $p70^{S6K}$. Very little is known about the links that connect cell surface receptors to the rapamycin sensitive steps. Use of Raf and Ras dominant negative mutants have indicated that they are not involved in the activation of $p70^{S6K}$ (Ming *et al*, 1994).

Work by *Chung et al*, including experiments with wortmannin have shown that PI 3-kinase was involved in $p70^{S6K}$ activation (Chung *et al*, 1994). Other evidence for its involvement has come from experiments showing that the synthesis of a constitutively active PI 3-kinase caused activation of $p70^{S6K}$ (Weng *et al*, 1995) and the involvement of protein kinase B (PKB). PKB can be activated by PI(3)P and activation of PKB by PDGF or insulin can be inhibited by wortmannin, suggesting that PKB lies downstream of PI 3-kinase (Franke *et al*, 1995; Kohn *et al*, 1995). Furthermore, activation of PKB is sufficient to activate $p70^{S6K}$, placing PKB upstream of $p70^{S6K}$ (Burgering & Coffer 1995). Along with the previous evidence that phorbol ester activation of $p70^{S6K}$ is not affected by wortmannin, this would suggest that PKB is either upstream of, or acts in parallel with the rapamycin sensitive step involved in activation of $p70^{S6K}$.

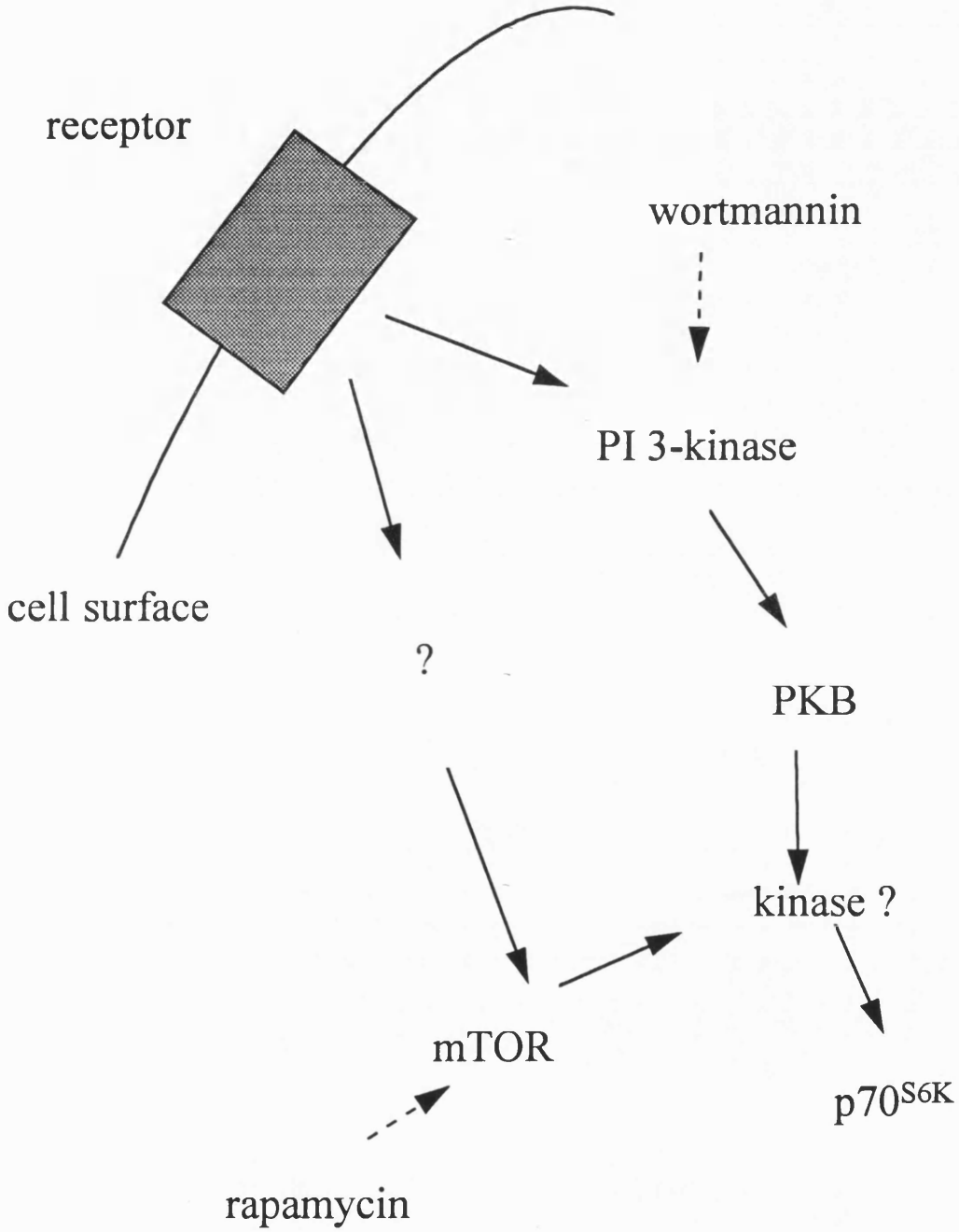


Fig. 1.4 Activation of $p70^{S6K}$

1.3.2.3 Serine/Threonine Protein Kinase Activity

PTK/SH2 coupled PI 3-kinase is a dual specificity kinase and not only phosphorylates phosphoinositides, but the p110 subunit can act as a serine/threonine protein kinase. To date, only two substrates have been identified : the p85 subunit, causing a decrease the lipid activity of the enzyme suggesting a form of auto-regulation, and insulin receptor substrate-1 (IRS-1) (Lam *et al.* 1994). The G protein sensitive PI 3-kinase has not been shown yet to possess serine/threonine kinase activity. This could potentially differentiate between the activation of the PI 3 - kinase pathway by G proteins versus its control by tyrosine kinases.

A full understanding of this family of PI 3-kinases is likely to take some time due to the complexity of the numerous interactions which appear to take place between the various signalling molecules.

1.3.3 RANTES and Signal Transduction

RANTES signalling is, as yet, poorly understood. All of the chemokine receptors identified belong to the superfamily of G protein linked receptors containing seven transmembrane spanning domains, which is consistent with the fact that many of the functions of these chemokines can be blocked by pertussis toxin. This includes the recently discovered C-C CKRs.

The C-C CKR1 is G protein linked and Ca^{2+} mobilisation is detected after MIP-1α, MCP-3 or RANTES stimulation (Combadiere *et al.* 1995b). However, some groups have observed RANTES stimulation of C-C CKR1 on monocytic cells inducing

migration without Ca^{2+} mobilisation, suggesting that a measurable Ca^{2+} mobilisation is not always necessary for the functional effects of RANTES and that another signalling pathway apart from the classical phosphoinositide pathway is involved (Van Riper *et al.* 1993). This is contradicted by others who were able to detect Ca^{2+} mobilisation (Wang *et al.* 1993). This can be explained by differences in experimental methods, e.g., the use or absence of serum in the vehicle, the use of different monocytic cells, THP-1 cell line or freshly prepared monocytes, and different methods for assessing migration.

There is also evidence to show that C-C CKR2 is G protein linked and activation of the receptor leads to calcium mobilisation (Myers *et al.* 1995). C-C CKR3 is pertussis toxin sensitive suggesting the involvement of a G_i protein (Kapp *et al.* 1994) and leads to intracellular Ca^{2+} mobilisation (Rot *et al.* 1992). The signalling pathways connected to C-C CKR3 have been studied further, and staurosporine, an effective inhibitor of the phospholipid/Ca^{2+} dependent PKC and wortmannin completely abrogated the production of reactive oxygen species induced by RANTES (Bourne *et al.* 1995; Kapp *et al.* 1994). This again illustrates the complexity of RANTES signalling. Finally, activation of the C-C CKR4 by RANTES induces a rapid change in intracellular Ca^{2+} levels, as well as histamine release and cell migration which are all pertussis toxin sensitive (Bischoff *et al.* 1993).

1.4 T Lymphocyte Activation and The Costimulatory Concept

The signals generated through engagement of the TCR by foreign antigen associated with self MHC (Ag-MHC) are not sufficient for full activation of T lymphocytes and the cells enter into a state of proliferative hyporesponsiveness (*in vitro* this is referred to as anergy and *in vivo*, tolerance), or the cells may undergo programmed cell death (PCD)(Bretscher, 1992). Another signal is required for IL-2 production and proliferation, giving rise to the two signal hypothesis, where engagement of the TCR, leading to PTK dependent activation of Ras and PLC signalling pathways, provides signal 1 (Pastor *et al.* 1995; Fraser *et al.* 1992; Metzner *et al.* 1994). Signal 2 is still not completely identified but there are several possibilities : i) it may be a unique signal activating distinct signal transduction pathways which complement those activated by TCR, ii) it may be the same as signal 1 but occur at different time points or in different cellular compartments, or iii) it may be the same as signal 1 but act in an additive fashion by enhancing the amplitude or duration of signal 1, and thus crossing a threshold and activating downstream signalling cascades.

Signal 2 is provided by costimulatory molecules on antigen presenting cells. The major costimulatory signal involved in T lymphocyte activation is provided by the interactions of the CD28 cell surface antigen on the T lymphocyte and its ligands, the B7 family, on the antigen presenting cell (APC) (Linsley *et al.* 1991a; Linsley *et al.* 1991b). This has been confirmed by the use of CD28-deficient mice where responses to mitogens were impaired, although T lymphocyte development was normal (**Fig. 1.5**)(Shahinian *et al.* 1993).

As predicted by the two signal hypothesis, CD28 ligation alone has little effect on resting T cell proliferation, but controls proliferation and IL-2 production from TCR stimulated CD28+ T cells (Linsley *et al.* 1991a). Costimulation via CD28 also mediates upregulation of other cytokines including IL-4, IL-8, IL-13, γ-interferon and granulocyte/macrophage colony stimulating factor (GM-CSF) (Bailey *et al.* 1994; Minty *et al.* 1993; Wechsler *et al.* 1994; Seder *et al.* 1994).

The cytoplasmic domain of CD28 does not have any intrinsic enzymatic activity and therefore must signal through the recruitment of cellular enzymes. This has analogy with TCR signalling where intracellular PTK such as fyn, lck and ZAP-70 are recruited. The cytoplasmic domain of CD28 contains a motif, $Y^{173}MNM$, similar to the SH2-binding motif, YXXM (Songyang *et al.* 1993), which is a potential site for binding of the SH2 domains present in the p85 regulatory subunit of PI 3-kinase, after tyrosine phosphorylation at position 173 by an unidentified kinase (Stein *et al.* 1994; Prasad *et al.* 1994; Truitt *et al.* 1994). CD28 also contains two proline rich motifs which conform to a SH3 binding consensus sequence and could also lead to interactions with PI 3-kinase (Johnson & Gomez-Cambronero, 1995). Many reports show association of CD28 with PI 3-kinase and site-specific mutagenesis of ^{173}Y to ^{173}P disrupts the SH2 binding motif and prevents costimulatory activity in some models (Ward *et al.* 1993; Pages *et al.* 1994; Sansom *et al.* 1995; Cai *et al.* 1995). Wortmannin has also been used to show that PI 3-kinase is pivotal in CD28 costimulation (Ward *et al.* 1995). However, other studies have revealed that PI 3-kinase is unimportant in CD28 costimulation (Truitt *et al.* 1995).

CD28 costimulation inhibits IL-2 mRNA degradation and can regulate many transcription factors e.g. NFκB, c-Rel and AP-1 (Rincon & Flavell, 1994). There is evidence to suggest that PI 3-kinase is the link between CD28 costimulation and the regulation of these transcription factors, since downstream targets of PI 3-kinase such as $p70^{S6K}$ (Downward, 1995), PKC isoforms (Hosaka *et al.* 1994; Toker *et al.* 1994) and ERKS and JNK (Pastor *et al.* 1995) are known to control transcriptional events.

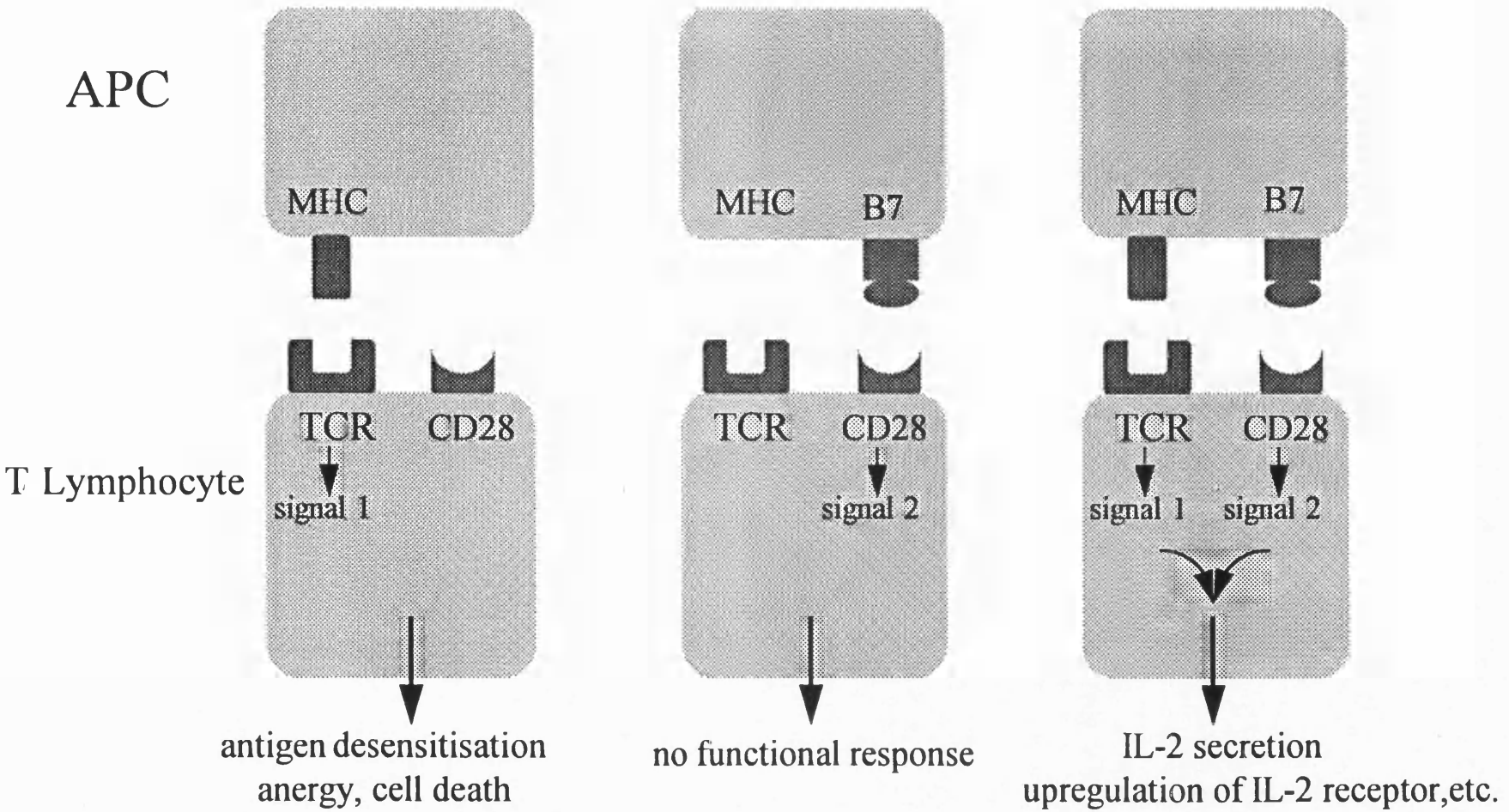


Fig. 1.5 Costimulation of T Lymphocytes by APC

1.5 Aims

The purposes of this study were to

a) examine the effects of RANTES on *in vitro* migration of T lymphocytes, not only analyzing migratory effects but looking at two effects that precede migration, shape change and actin polymerisation. To this end, T lymphocytes from several sources were used - primary T lymphocytes, T lymphoblasts and the Jurkat T cell line.

b) investigate the role of the classical phosphoinositide signalling pathway in RANTES stimulation of T lymphocytes by analyzing calcium mobilisation and PtdIns metabolism.

c) evaluate the involvement of the PI 3-kinase signalling pathway in RANTES stimulation of T lymphocytes by analyzing PtdIns metabolism, in particular the effects RANTES has on the activity of PI 3-kinase and the production of D-3 phosphoinositides, and by using the PI 3-kinase inhibitor, wortmannin.

d) assess the role RANTES plays in T lymphocyte functions, other than migration, such as proliferation, IL-2 receptor upregulation and IL-2 production.

SECTION TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Culture Reagents

Plastics (sterile, cell culture grade) were purchased from Becton Dickinson (Plymouth, U.K.). Heparin (Monoparin™, heparin sodium (mucous), 5000 units/ml) was purchased from FISONS (Loughborough, U.K.) and stored at 4°C. RPMI 1640, sterile culture medium, was purchased from Life Technologies Inc. (Paisley, U.K.), supplemented with 5 μg/ml fungizone and 20 Units/ml of penicillin and streptomycin, and stored at 4 °C in the dark. Lymphoprep™, specific gravity of 1.077, was purchased from Nycomed (Birmingham, U.K.) and stored in the dark at 4 °C. Phosphate buffered saline (PBS)(sterile, without calcium and magnesium, 10X) was bought from Life Technologies Inc. (Paisley, U.K.), stored at room temperature and diluted to 1X with sterile water. Heat inactivated bovine serum (HIBS) was either purchased from Life Technologies Inc. (Paisley, U.K.) already heat inactivated or non-inactivated serum, from the same source, was inactivated by heating at 56 °C for 30 minutes, then stored at -20 °C. R+D human T cell enrichment columns were purchased from R+D (Abingdon, U.K.). Phytohaemagglutinin (PHA-P) was purchased from Sigma (Poole, U.K.), and made up to a 1 mg/ml sterile solution with sterile water, then stored at - 20°C. Trypsin/EDTA (0.5 mg/ml trypsin, 0.2 mg/ml EDTA) was purchased from Life Technologies Inc. (Paisley, U.K.) and stored at - 20 °C. Dulbecco's modified Eagle's medium (DMEM), sterile culture medium, was purchased from Life Technologies Inc. (Paisley, U.K.), supplemented with 5 μg/ml fungizone and

20 Units/ml of penicillin and streptomycin, and stored at 4 °C in the dark. Hank's balanced salt solution (HBSS, sterile, 10X) was purchased from Life Technologies Inc. (Paisley, U.K.). A 1X solution was prepared by adding 900 mls sterile water to the 100 mls 10X solution, then 4.9 mls sterile 7.5 % sodium bicarbonate solution was added to give a pH of 7.3.

The preparation of cells and sterile experiments were all performed in class 2 sterile cabinets. The cell cultures were incubated at 37 °C in a humidified 5% CO_2 incubator.

2.1.2 General Reagents

Polyvinylpyrrolidine (PVP) free polycarbonate filter (8 μm pore size) membranes were purchased from Costar (High Wycombe, U.K.) Diff-Quik was purchased from Browne (Newbury, U.K.). FITC-phalloidin (fluorescein thiocyanate conjugated phalloidin) was purchased from Sigma (Poole, U.K.), and made up to a stock solution of 30 μM in sterile PBS, then stored in the dark at - 20 °C. Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, USA) and stored at 5 mM in anhydrous DMSO, at - 20 °C, in the dark. Protein A Affigel beads were purchased from BIO-RAD (Hemel Hempstead, U.K.) and stored at 4 °C. Wortmannin was dissolved in ethyl acetate to a concentration of 20 mM and stored in aliquots at - 20 °C in the dark. Before use, the stock was diluted in culture medium or buffer, to the desired concentrations. Scintillation fluid (Optiphase) was purchased from FISONS (Loughborough, U.K.).

All solvents were purchased from FISONS (Loughborough, U.K.)

All other chemicals were bought from Sigma (Poole, U.K.), unless otherwise stated.

2.1.3 Antibodies and Cytokines

IL-2 was purchased from Genzyme (West Malling, U.K.) and stored at 20 μg/ml in sterile RPMI 1640, at 4 °C. Before use, the stock was diluted in culture medium to the desired concentrations. UCHT1, mouse anti-human CD3, was a kind gift from Doreen Cantrell (ICRF, London, U.K.). UCHT1 was stored in sterile PBS at 1 mg/ml. Long term storage of UCHT1 was at - 70 °C , while the working aliquots were stored at 4 °C. Before use, the stock was diluted in culture medium to the desired concentrations. Goat anti-mouse IgG (conjugated to phycoerythrin) was purchased from Dako (High Wycombe, U.K.) and stored at 4 °C. Mouse anti-human CD4, CD8, CD11, CD16, CD19, CD25 (FITC-conjugated), CD45RO, CD45RA were all purchased from Sigma Immunochemicals (Poole, U.K.) and stored at 4 °C. Human RANTES was purchased from Peprotech (Rocky Hill, NJ, USA) and stored at 100 μg/ml at - 20 °C in sterile water. Before use, the stock was diluted in culture medium to the desired concentrations. Rabbit polyclonal anti-human PI 3-kinase was purchased from UBI (New York, NY, USA) and stored at - 20 °C. anti-human RANTES, mouse monoclonal and goat polyclonal, were both purchased from R+D (Abingdon, U.K.) and stored at 500 μg/ml in sterile PBS/0.1 % BSA, at - 20 °C.

2.1.4 Radioisotopes

[γ-^{32}P] labelled ATP ([^{32}P]-ATP) (5 μCi/ml, 3000 Ci/mmol, in aqueous solution) and [^{32}P] labelled orthophosphate ($^{32}P_i$) (5 mCi/ml, 8500 - 9120 Ci/mmol) were purchased from DuPont NEN (Stevenage, UK). [^{3}H] thymidine (1 mCi/ml, 2 Ci/mmol) was purchased from Amersham (Little Chalfont, U.K.).

2.1.5 Solutions

The composition of CHO-DMEM, 1X NP40 lysis buffer and lipid kinase buffer were as follows :

CHO-DMEM - 100 ml 10X DMEM

100 ml HIBS

50 ml sodium bicarbonate (7.5 %)

25 ml 1 M HEPES

20 ml nucleoside solution [1 mM adenosine, cytidine, uridine, thymidine, and guanosine in sterile water, sterile filtered before use]

and 800 ml sterile H_2O.

Stored at 4 °C for up to two months.

1X NP40 lysis buffer - 1% NP40 solution

100 mM NaCl

10 mM iodoacetamide

10 mM NaF

10 mM Tris pH 7.4 in sterile H_2O

Stored at 4 °C for up to two months.

lipid kinase buffer - 5 mM $MgCl_2$

0.25 mM EDTA

20 mM HEPES (sodium salt) pH 7.4 in sterile H_2OStored

Stored at 4 °C for up to two months.

2.2 Human Peripheral Blood Mononuclear Cell Preparation

Human peripheral blood mononuclear cells (PBMC) were isolated from venous blood which had been collected from healthy donors of either sexes. The blood was collected from donors aseptically into a 50 ml sterile disposable syringe using a " butterfly " cannula (19 - 23 gauge needle). Sterile heparin was used as an anti-coagulant (10 Units heparin/ml blood). Alternatively, whole blood packs were purchased from the Blood Transfusion Service (Bristol, U.K.). The blood was decanted into sterile tubes, centrifuged (1860 g, 6 min, 4°C), and the plasma was removed with a sterile plastic pipette (Bacon *et al.* 1988).

The blood was then diluted four-fold with RPMI 1640 and gently mixed in a sterile 75 cm^2 flask. The diluted blood (35 ml) was layered onto 15 ml Lymphoprep ™ in sterile 50 ml tubes at room temperature. Mononuclear cells were separated by centrifugation (460 g, 30 min, 20°C). The mononuclear cell layer was removed using a sterile pastette. Aliquots of 15 ml mononuclear cell suspension were placed in sterile 50 ml tubes and 30 ml sterile phosphate buffered saline (without calcium and magnesium) (PBS) was added to each tube. The tubes were centrifuged at 450 g for 7 minutes, room temperature, and the supernatants discarded. The cell pellet was washed twice by resuspension in 50 ml RPMI 1640 and centrifugation at 450 g for 7 minutes, at room temperature.

2.3 Primary T Lymphocyte Purification

It was important to obtain purified T lymphocyte populations with no contaminating monocytes or B lymphocytes so two different methods of purifying the PBMC were attempted.

2.3.1 Primary T Lymphocyte Purification Using Plastic Adherence

The viability of the PBMC was assessed by Trypan blue exclusion before they were resuspended at a cell concentration of 5 x 10^6 cells/ml, in RPMI 1640 supplemented with 10% heat inactivated bovine serum (HIBS). The cells were aliquoted (5 ml) onto sterile petri dishes to allow cell adherence to the plastic dish and incubated for one hour, after which the non-adherent cells were removed from the dishes using a sterile pastette and washing with sterile PBS three times (Bacon *et al.* 1988). These cells were resuspended at 2 x 10^6 cells/ml in RPMI 1640 supplemented with 10% HIBS in a sterile 75 cm^2 culture flask and incubated for 18 hours, to ensure removal of all adherent cells. The non-adherent cells were washed in RPMI 1640 and the cell viability was determined again using Trypan blue exclusion. The purified T lymphocytes were resuspended at 2 x 10^6 cells/ml in serum-free RPMI 1640, before assay.

2.3.2 Primary T Lymphocyte Purification Using A Human T Cell Enrichment Column

Freshly isolated PBMC were prepared as before (2.2) and resuspended in 2 ml 1X wash buffer provided by column manufacturer. The column was clamped on to a stand and the column fluid was allowed to drain into a waste receptacle. The column was then equilibrated by washing with 8 ml of 1X column wash buffer and the eluate allowed to drain into the waste receptacle. A sterile 50 ml tube replaced the waste receptacle and

the 2 ml cell suspension was applied to the top of the column. This replaced the wash buffer contained in the column, which was collected in the sterile centrifuge tube. The cells, which were now retained in the column, were incubated at room temperature for 10 minutes.

After this incubation step the cells were eluted from the column with 15 ml of column wash buffer. The collected cells were washed in RPMI 1640 and centrifuged at 450 g for 7 minutes. The supernatant was decanted and the cells resuspended in RPMI 1640. The cell viability was checked using Trypan blue exclusion and the purified T lymphocyte were resuspended at 2 x 10^6 cells/ml in RPMI 1640 before assay.

2.3.3 Analysis of T Lymphocyte Purity

The primary T cell populations prepared in 2.3.1 and 2.3.2, were analyzed for contaminating cells by either examining the populations on cytospins morphologically or by FACS analysis. 1 x 10^5 purified T cells were incubated with 100 μl either mouse anti-human CD3 (T lymphocyte marker), or mouse anti-human CD11 (monocyte marker), or mouse anti-human CD16 (NK cell marker) or mouse anti-human CD19 (B cell marker), (1:50 dilution in RPMI 1640), as primary antibodies, for 30 minutes at 4°C. The cells were washed with 5 ml sterile PBS and then incubated with 100 μl secondary antibody, goat anti - mouse IgG conjugated to phycoerythrin, (1:50 dilution in RPMI 1640), for 30 minutes at 4 °C. The cells were washed with 5 ml sterile PBS and then analyzed on a Becton Dickinson FACS vantage (excitation wavelength - 488 nm, emission wavelength - 575 $\pm$ 13 nm).

2.3.4 Analysis of T Lymphocyte Subsets

The T lymphocyte subsets present in the primary T lymphocyte populations prepared in 2.3.1 were identified by a similar method to 2.3.3, although this time the primary antibodies used were mouse anti - human CD4, CD8 , CD45RO, CD45RA.

2.4 Human T Lymphoblast Preparation

Human T lymphoblasts were prepared by stimulation of PBMC with 1 μg/ml phytohaemagglutinin (PHA) in RPMI 1640 plus 10% HIBS for 72 h to promote IL-2 receptor expression. After 72 h, the non - adherent lymphocytes were washed three times in RPMI 1640 and maintained in culture with RPMI 1640 plus 10% HIBS supplemented with 20 ng/ml human interleukin-2 (IL-2) every 48 hours for 10 - 14 days. T lymphoblasts were passaged every 2 days by 1:2 dilution in RPMI 1640 plus 10% HIBS and 20 ng/ml IL-2 (Smith & Cantrell, 1985).

T lymphoblasts were sometimes quiesced into the resting phase of the cell cycle (G_0/G_1) before use. This involved removing the cells from culture, washing in RPMI 1640 and resuspending in RPMI 1640 plus 10% HIBS without IL-2 for approximately 24 hours. This ensures that all cells were synchronized into the resting phase of the cell cycle.

2.5 Maintenance of Cell Lines

The leukaemic Jurkat cell line (J6 subclone) was passaged by 1:10 dilution every three days, in RPMI 1640 supplemented with 10% HIBS. These cells were cultured in 175 cm^2 sterile culture flasks and maintained at 37 °C, 5 % CO_2. The viability of cell lines was checked using Trypan blue exclusion before use in all assays.

Chinese hamster ovary cells (CHO) and CHO transfected with B7 (CHO-$B7^+$)(both gifts from D.Sansom, BIRD, Bath, U.K.) were maintained in CHO-DMEM (see 2.1.5) in 175 cm^2 culture flasks. The adherent CHO cells were passaged by 1:10 dilution every three days. This involved decanting the media and washing the adherent cells with 50 ml sterile PBS which was then discarded into a waste receptacle. The CHO cells were trypsinised with 2 ml Trypsin - EDTA /175 cm^2 for 5 minutes at 37 °C, 5 % CO_2 and removed from the flask into 50 ml CHO-DMEM. 2 ml cell suspension was added to each new flask and supplemented with 20 ml CHO-DMEM. Alternatively the trypsinised cell suspension was used in experimental procedures. If they were to be used as antigen presenting cells, the cells required to be fixed as follows : the cell suspension was centrifuged at 450 g for 5 minutes and the cells were fixed at a cell concentration of 1 - 5 x 10^6 cells/ml, in 1 ml sterile filtered 0.1 % solution of glutaraldehyde in PBS. The cells were fixed in glutaraldehyde for 2 minutes, before 2 ml of 200 mM glycine (sterile filtered) was added for 2 minutes. 40 ml RPMI 1640 was added and the cells were centrifuged at 450 g for 5 minutes, before resuspension in media at the appropriate cell concentration required for the assay.

2.6 Chemotaxis Assay

Purified T lymphocyte, T lymphoblast and the Jurkat T cell line migration after stimulation with certain chemoattractants was examined using a 48-well microchemotaxis chamber (Neuro Probe)(Cabin John, MD, USA).

The chamber was oriented so that the trademark was always kept in the upper left corner. The lower wells were filled with either 25 μl chemoattractant in RPMI 1640 that had been warmed to 37 °C or warmed RPMI 1640 only, as a vehicle control. 1 mm of the corner of a polyvinylpyrrolidine (PVP) free polycarbonate filter (8 μm pore size) membrane was cut off, enabling easier identification of the orientation of the membrane (Bacon *et al.* 1988). The filter was lifted carefully and placed carefully over the lower wells with the cut corner kept in the upper left corner. The silicone gasket was applied with the cut corner at the upper left, then the top plate with the trademark also oriented at the upper left. The top plate was tightened on with the screws then the upper wells were covered with a glass microscope slide, to stop evaporation of the chemoattractant while the chamber was allowed to equilibrate (5% CO_2, 30 minutes, 37°C). 50 μl of cell suspension in RPMI 1640 at 2 x 10^6 cells/ml was placed in each of the upper wells, making sure that no air bubbles were trapped in the wells. The upper wells were again covered with the glass microscope slide and left at 5% CO_2, 37 °C (1 - 5 hours, see legends)(**Fig. 2.1**).

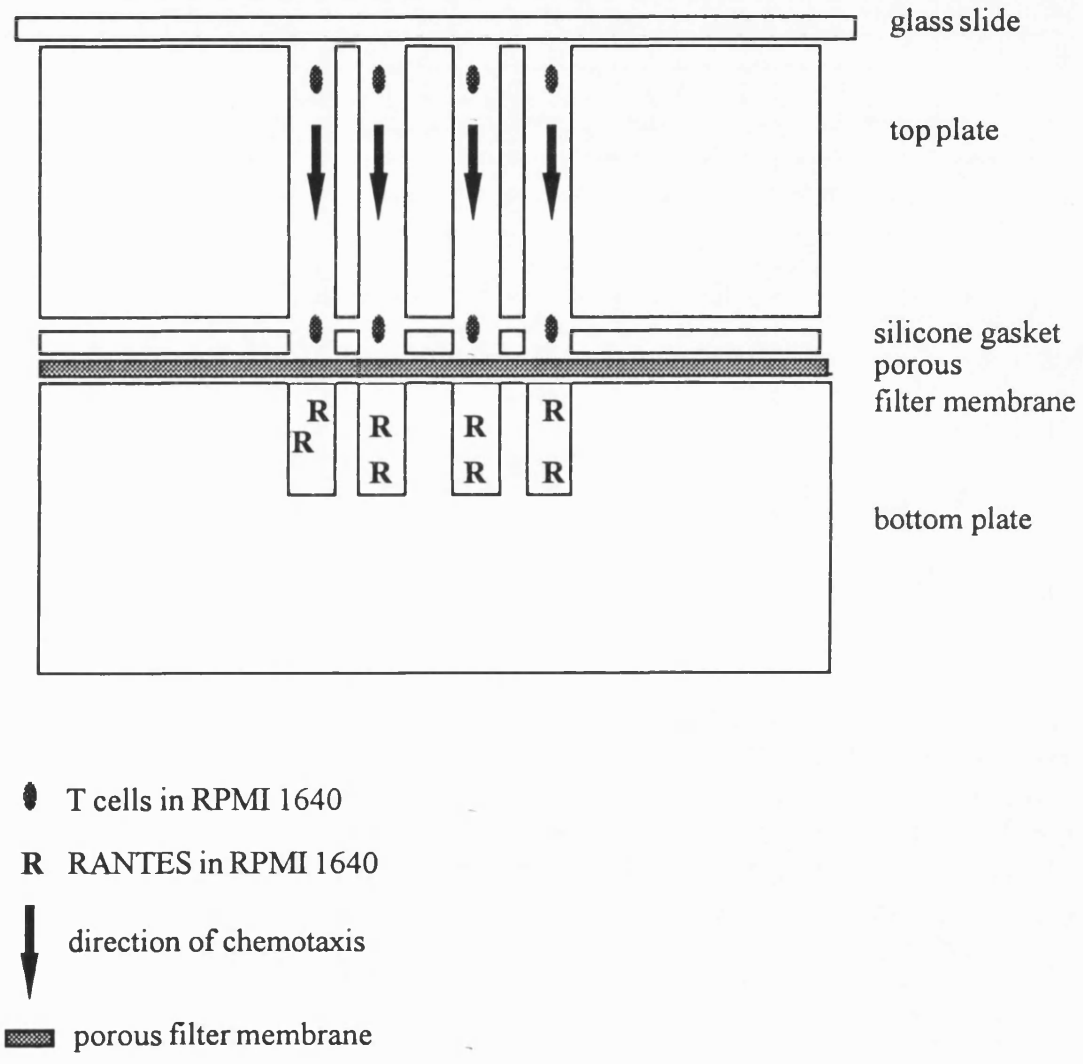


Fig. 2.1 Schematic Diagram of the 48-Well Microchemotaxis Assay - transverse cross-section

After incubation, the screws were removed and the whole chamber was inverted onto a paper towel. The bottom plate was detached carefully from the upper plate and the silicone gasket. The inversion of the whole chamber meant that the cells that had migrated, and had therefore moved through the pores of the membrane, were now on the surface of the membrane which was now uppermost. The filter was removed and the non-cell side (the underneath side) was thoroughly washed three times in sterile PBS. The filter was allowed to dry then fixed in methanol for 2 minutes and stained with Diff-Quik. The wet membrane was placed on a microscope slide, allowed to dry and lymphocyte migration was assessed by counting the number of cells attached to the lower surface of the filter membrane, in five high power (400 x) magnification fields for each well.

2.7 Polarisation Assay

Before T lymphocytes migrate in response to a chemoattractant the cells undergo several changes after activation including a change in morphology from spherical T lymphocytes to a more polarised shape.

The polarising response of purified T lymphocytes to chemoattractant was analysed by detecting morphology changes in the cells (Haston & Shields, 1985; Newman & Wilkinson, 1993). 3 x 10^6 purified T lymphocytes were washed three times with sterile Hank's Balanced Salt Solution containing 10 mM morpholinopropane sulphonic acid (pH 7.4)(HBSS/MOPS) and resuspended in 1.5 ml HBSS/MOPS in 6 ml polystyrene tubes. The cells were then incubated with chemoattractant or vehicle control at 5 % CO_2, 37 °C. The incubation times were varied to find an optimal incubation time (30 -

240 minutes).

The reaction was stopped by the addition of 1.5 ml 2.5 % glutaraldehyde in sterile PBS for 15 minutes and the cells were washed in HBSS/MOPS three times. The samples were then resuspended in 0.5 ml HBSS/MOPS. The extent of polarisation was assayed by counting the number of polarised cells compared to cells with a spherical shape in a total count of 300 purified T lymphocytes, under 400 x magnification for each sample.

2.8 Actin Polymerisation Assay

Another change that occurs after T lymphocytes are activated by a chemoattractant is a change in the actin structure in the cytoskeleton of the cells. T lymphocytes in the resting state have globular actin (G - actin). When they are activated this G - actin polymerises to filamentous actin (F - actin). This can be detected using FITC - phalloidin which will only bind to the F - actin, not G - actin, and as it is fluorescent this can be detected using FACS analysis.

0.5 ml purified T lymphocytes at 2 x 10^6 cells/ml in RPMI 1640 were stimulated with chemoattractant or vehicle control (10 μl) in a 24 well plate, 5 % CO_2, 37 °C. The reaction was quenched by removing the cell suspension from the plate with a pastette into FACS tubes containing 0.5 ml of 7.4 % formaldehyde in PBS solution. The cells were left for 10 minutes at room temperature to fix. Excess PBS was added and the cells were spun for 7 minutes at 500 g. The pellet was resuspended in 100 μl of 1 % formaldehyde in PBS solution supplemented with 1 % HIBS. 100 μl of 0.3 μM FITC -

phalloidin in 0.1 % triton/ PBS was added to the cell suspension and the cells were left to incubate at 4 °C for 30 minutes. The cells were then washed twice in PBS and resuspended in 500 μl of 1 % formaldehyde in PBS solution supplemented with 1 % HIBS. The samples were stored in the dark at 4 °C until analyzed. They were analyzed on a Becton - Dickinson FACS vantage, excitation wavelength - 488 nm, emission - 530 $\pm$ 15 nm (Personal communication with Dr S. Kellie, Yamanouchi, U.K.).

2.9 Determination of Cytosolic Free Calcium $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ in human T lymphoblasts, the Jurkat cell line and purified T lymphocytes was measured by monitoring the fluorescence of these cells loaded with the calcium indicator, fura-2 acetoxymethyl ester (fura-2). This compound is lipophilic so can penetrate the cell membrane and pass into the cell interior where the ester groups are cleaved trapping the indicator in the cell cytosol. Fura-2 undergoes a spectral shift upon binding Ca^{2+} and by using the ratio of fluorescence intensities at two separate excitation wavelengths, as opposed to only using one excitation wavelength, differences between loading with the dye on different days is taken into account, and we can calculate the $[Ca^{2+}]_i$ and compare $[Ca^{2+}]_i$ elevations from different experiments.

The cells were resuspended at 10^7 cells/ml in RPMI 1640 supplemented with 10% HIBS in 50 ml sterile tube. The cells were incubated with 2.5 μM fura-2 acetoxymethyl ester for 30 minutes, 37 °C, in the dark (Ward *et al.* 1988). The cells were washed three times in 50 ml of HBSS/BSA (pH 7.4, 0.25 % bovine serum albumin) and finally resuspended at 2 x 10^6 cells/ml in HBSS/BSA. The cell suspension was kept in the

dark at all times.

Aliquots (2 ml) were dispensed into a quartz cuvette and the external calcium concentration adjusted to 1 mM with $CaCl_2$. The $[Ca^{2+}]_i$ was determined by recording the fluorescence signals of these stirred aliquots at 37°C in a fluorimeter (slit widths - 4 nm), with dual excitation wavelengths (340 nm and 380 nm) and a single emission wavelength of 510 nm before and after appropriate stimuli.

Cells were lysed with 125 μM digitonin (added from a 4 mg/ml digitonin stock made up in 70% ethanol) to give the maximum fluorescence signal (R_{max}). The fluorescence level in Ca^{2+} free medium (R_{min}) was calculated by adjusting the pH of the cell lysate to > 8.5 with NaOH (40 μl of 4M NaOH solution) and then adding 10 mM EDTA to reduce the extracellular free concentration to < 10 nM. R_{max} and R_{min} were then used in the ratio method with PTI software (Grynkiewicz *et al.* 1985). In brief, conversion of the sample fluorescence ratio (R) to intracellular free calcium levels ($[Ca^{2+}]_i$) were made using a simple formula, where R is the sample fluorescence ratio in arbitrary units and K_d is the dissociation constant of the Ca^{2+} - fura-2 complex which is 224 nM (Grynkiewicz *et al.* 1985) :

$$[Ca^{2+}]_i = (R - R_{min}/ R_{max} - R) * K_d$$

Fura-2 loaded cells were allowed to equilibrate for 2 minutes at 37 °C before drugs or vehicles were added directly to the cuvette containing the cells. The quartz cuvette was washed thoroughly between each sample.

2.10 Phosphoinositide 3-kinase Activity

One signal pathway relevant to chemotaxis may be the putative pathway regulated by phosphoinositide 3-kinase (PI 3-kinase) and its metabolic products, D-3 phosphoinositide lipids. One approach is to examine the production of the D-3 phosphoinositides in this enzymatic pathway. Cells are radiolabelled with [^{32}P]-orthophosphate and stimulated with appropriate stimuli. The phosphoinositides are then extracted and analysed using High Performance Liquid Chromatography. Alternatively, PI 3-kinase activity can be determined, after stimulation, by immunoprecipitating the enzyme with anti - human PI 3-kinase coupled to Affigel beads, and then performing an *in vitro* lipid kinase assay with an exogenous substrate and [γ-^{32}P] labelled ATP, on the immunoprecipitates. The resulting radiolabelled phosphoinositides are then visualised using thin layer chromatography.

2.10.1 *In Vitro* Measurements of Phosphatidylinositol Lipid Metabolism

Purified T lymphocytes, Jurkat cells or T lymphoblasts were washed three times in phosphate free (Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM HEPES, pH 7.2), with a 10 minute incubation at 37°C, between each wash. Cells were resuspended in 5 ml phosphate free DMEM , 20 mM HEPES and 10 % dialysed HIBS (dialysed against saline).

After incubation with 1 mCi of [^{32}P] labelled orthophosphate ($^{32}P_i$) for 4 hours at 37°C, the cells were washed three times in phosphate free DMEM and resuspended in RPMI 1640. 0.5 ml aliquots were dispensed into eppendorf tubes and treated with appropriate stimuli at 37°C. The reactions were quenched with 0.5 ml

chloroform/methanol/distilled H_20 (32.65%/65.3%/2.15%(v/v)) and maintained on ice for 10 minutes (Jackson *et al.* 1992). Phases were separated by addition of 200 μl chloroform (containing 20 μg Folch lipids) and 200 μl 2.4M HCl, 5 mM tetrabutylammmonium sulphate. The samples were vortexed and centrifuged (16000 g, 5 minutes, 4°C). The lower phase was removed carefully into clean eppendorf tubes containing 0.5 ml synthetic upper phase (0.1 M HCl, 5 mM tetrabutylammonium sulphate, 25 mM EDTA). The phases were again mixed by vortexing and centrifuged (16000 g, 5 minutes, 4°C) and the lower phase removed into clean tubes then dried *in vacuo* (Savant speedvac).

The samples were then deacylated by adding 1 ml 25% w/v methylamine in water/ methanol/butanol (44.4%/44.4%/11.1%). Samples were vortexed, incubated (40 minutes, 53°C) and subsequently cooled on ice for 5 minutes and dried *in vacuo*. 0.5 ml sterile water was added to the dried samples, followed by 0.6 ml n-butanol/40-60% petroleum ether/acetate (80%/16%/4% v/v). After vortexing and centrifuging (16000 g, 30 seconds, 23°C) the upper phase was discarded and the lower phase dried *in vacuo*.

The resulting pellets were resuspended in 120 μl sterile water in a sonicating bath. The deacylated lipids were separated by high performance liquid chromatography (HPLC) on a partisphere-SAX column using a gradient based on buffers A (ddH_2O)/B [1.25 M $(NH_4)_2HPO_4$)](pH 3.8) at a flow rate of 1.0 ml/minute: 0 minute, 0 % B; 5 minutes , 0 % B; 45 minutes, 12 % B; 60 minutes, 30 % B; 61 minutes, 100 % B; 65 minutes, 100 % B; 66 minutes, 0 % B (Ward *et al.* 1992a). The eluate was fed into a Canberra Packard A-500 Flo-One on-line *beta*-radiodetector where it was mixed with three parts

Flo-Scint IV scintillation cocktail and the results analyzed on the Flo-one data program (Radiomatic, USA).

2.10.2 Preparation of Protein A Affigel Beads

500 µl 50 % suspension of Protein A Affigel beads in PBS was placed in a clean eppendorf tube and washed three times in PBS. After the third wash all the PBS was removed with a Hamilton syringe. 25 µl rabbit polyclonal anti-human PI 3-kinase was added to the beads and 1 ml PBS added, to fill the eppendorf tube. The tube was then rotated at room temperature for 2 hours. Finally, the antibody coupled beads were washed three times with PBS and resuspended in PBS to 500 µl to give a 50 % suspension. The beads were stored at 4 °C until ready to be used.

2.10.3 Immunoprecipitation of PI 3-Kinase

The purified T lymphocytes were resuspended at 4 x 10^6 cells/ml in RPMI 1640. 1 ml cell suspension was stimulated with appropriate treatment. The cells were spun down into pellets (450 g, 7 minutes). 50 ml 1X NP40 lysis buffer (see 2.1.5) was supplemented with 46 mM ß-glycerophosphate, 1 µg/ml leupeptin, 1 µg/ml chymostatin, 1 µg/ml antipain, and 1 µg/ml pepstatin A before use and the lysis buffer was kept on ice (Ward *et al.* 1992b). The cells were resuspended in 1 ml ice cold 1X NP40 lysis buffer and transferred to eppendorf tubes containing 10 µl phenylmethylsulfonyl fluoride (PMSF). These eppendorf tubes were placed in a rotator and left to rotate for 15 minutes at 4 °C.

The lysates were then precleared, to reduce non-specific binding of the beads to the cell lysates. The cell lysates were removed to clean eppendorf tubes containing 40 μl uncoupled protein A bead solution and rotated for 1 hour at 4 °C, after which time the beads were spun down (450 g, 10 minutes) and the uncoupled lysates were again removed to clean eppendorf tubes, this time containing 20 μl rabbit anti-human PI 3-kinase coupled to Protein A bead solution. The lysates were left to rotate for 2 hours at 4 °C. Then the coupled beads were pelleted at 450 g for 10 minutes. 20 μl cell lysate was kept as a control in a separate clean eppendorf tube and the anti - PI 3-kinase beads now coupled to the PI 3-kinase enzyme were then used in a kinase assay.

2.10.4 *In Vitro* Lipid Kinase Assay

The immunoprecipitates, from 2.10.3, were washed thoroughly. Three washes in 1 ml 1X NP40 lysis buffer, single washes in 1 ml PBS, 1 ml LiCl (0.5 M in H_2O), and 1 ml H_2O. Between each wash the beads were pelleted at 450 g for 10 minutes and all the wash buffer, except for 100 μl containing the beads, was removed by aspiration . After the last H_2O wash the beads were transferred to clean eppendorf tubes. The final wash was with 1 ml lipid kinase buffer (see 2.1.5). After pelleting, this wash buffer was removed completely with a Hamilton syringe and the pellet was resuspended in 80 μl lipid kinase buffer, 100 μl substrate (1 mg phosphatidylinositol and 1 mg phosphatidylserine in 1 ml 25 mM HEPES/1 mM EDTA, sonicated into solution) (Ward *et al.* 1992b). The assay was initiated by adding 20 μl [γ-^{32}P]ATP/cold ATP (200 μl lipid kinase buffer, 7 μl [γ-^{32}P]ATP, 2 μl 100 mM cold ATP in 100 mM Tris pH 7.0) to each eppendorf tube including the control eppendorf tube, and then leaving the tubes at room temperature for 15 minutes. The reaction was quenched with 200 μl

1N HCl and 400 μl chloroform:methanol (1:1), then the eppendorf tubes were spun at 16000 g for 30 seconds. This separated the phases and the lower organic phase containing the lipids was removed with a gel loading tip into clean eppendorf tubes. This phase was washed with 1 ml 1:1 methanol/HCl and the organic phases separated again by centrifugation at 16000 g for 2 minutes. The lower phase was then removed and dried *in vacuo*.

When the samples were dry they were resuspended in 50 μl chloroform. The samples were then spotted onto a potassium oxalate (1%) sprayed thin layer chromatography plate, 10 μl at a time, drying between each spot. Phospholipid standards, e.g., PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, were also added. Each lane was clearly labelled, then the plate was placed in an equilibrated thin layer chromatography (TLC) tank containing 130 ml propan-1-ol, 66 mls H_2O and 4 mls glacial acetic acid with a piece of 3M paper as a support for the solvent. The TLC plate was allowed to stand upright in the tank overnight.

The plate was removed from the tank and allowed to dry in a fume hood, then placed in an iodine tank to visualise the phospholipid substrates and standards. When the plate was dry again it was exposed to film using intensifying screens and left at - 80 °C until ready to develop.

2.10.5 Wortmannin Inhibition

Wortmannin, the PI 3-kinase inhibitor was used in many of the assays to assess the involvement of PI 3-kinase. Purified T lymphocytes were incubated with the wortmannin solutions or vehicle control for 10 minutes, 5 % CO_2, 37 °C. The cells were then stimulated with chemoattractant and the assays performed as normal. The final concentration of ethyl acetate was less than or equal to 0.005 %. Control migration experiments were performed with ethyl acetate only, at concentrations equivalent to the ethyl acetate concentrations present in the appropriate wortmannin solutions.

2.11 Proliferation Assay

The ability of lymphocytes to proliferate is a useful measure of lymphocyte function. Proliferation can be measured by the incorporation of [^{3}H]-thymidine into T lymphocyte DNA. The purified T lymphocytes were resuspended in RPMI 1640 plus 10% HIBS at 2.5 x 10^5 cells/ml. 190 μl T lymphocyte suspension was aliquoted into the wells of a 96-well, flat bottomed microtitre plate. Quadruplicate aliquots were treated with 10 μl vehicle, UCHT1, fixed CHO-B7$^+$, RANTES, mouse monoclonal anti-human RANTES, or various combinations of these stimuli. Each treatment was optimised using a range of concentrations.

The cells were then incubated for 72 hours at 37 °C, 5 % CO_2. The cells were pulsed with 0.5 μCi/well [^{3}H] thymidine for the final 18 hours of culture. The cells were then harvested onto Whatman GF/A filters using a multiple automated cell harvester. The incorporation of [^{3}H]-thymidine was measured using standard liquid scintillation

counting techniques. The radioactivity collected on the filter paper was assayed by placing it in 4 mls of scintillation fluid (Optiphase) and counted on a ß-scintillation counter (LKB). Results are expressed as counts per minute (cpm).

2.12 RANTES Production and Quantitation

The production of RANTES by purified T lymphocytes stimulated with various treatments can be measured using a double ligand sandwich Enzyme-Linked-Immunosorbent-Assay.

490 μl purified human T lymphocytes at 2 x 10^6 cells/ml in RPMI 1640 supplemented with 10 % HIBS were aliquoted into multiwell culture plates and incubated at 37 °C, 5 % CO_2. UCHT1, CHO-B7$^+$ and vehicle additions were made at 10 μl to give a final volume of 500 μl and incubated at 37 °C, 5 % CO_2 for 0 - 96 hours. After incubation, the cells were pelleted (450 g, 7 minutes) and the cell free supernatants were removed by suction, sub-aliquoted and stored frozen at - 20 °C.

A 96 well culture plate was coated with mouse monoclonal anti-human RANTES, 50 μl/well overnight at 4 °C at a final concentration of 1 μg/ml in coating buffer (25 mM Na_2CO_3, 30 mM $NaHCO_3$ in distilled water, pH 9.6). The plate was washed three times with buffer (PBS/0.05 % Tween 20, pH 7.5) to remove any unbound antibody. Diluted standards (6, 4, 3, 2, 1, 0.5, 0.25, 0.125, 0.63 ng/ml) and samples in wash buffer plus 2 % HIBS were loaded in duplicate, 50 μl/well. The first four wells were loaded with buffer only. The covered plate was incubated at 37 °C for 2 hours.

Goat polyclonal anti-human RANTES was biotinylated by dissolving RANTES in 100 mM HEPES, pH 8.5, at a concentration of 2 mg/ml. 10 μl of 20 mg/ml biotin (long arm) N hydroxysuccinimide ester (BNHS) in N,N-dimethylformamide (DMF) was added. With occasional stirring, the peptide was incubated at room temperature for 2 hours. The reaction was stopped by adding 10 μl triethanoloamine. The biotinylated protein was then dialysed against three changes of 2 litres of PBS.

The plate was washed three times in wash buffer and 50 μl of 1 μg/ml biotinylated goat anti-human RANTES in wash buffer plus 2 % HIBS was added to each well and incubated for 1 hour at 37 °C. The unbound biotinylated antibody was removed by washing three times in wash buffer. The enzyme streptavidin peroxidase was then added, 50 μl/well at 0.5 μg/ml in wash buffer. The plate was covered and incubated at 37 °C for 30 minutes. The plate was then washed three times in wash buffer. The substrate used was 1,2 phenylene dihydrochloride (OPD). This is oxidised to produce a coloured end product. 100 μl substrate was added to each well. The plate was allowed to incubate in the dark at room temperature for 25 minutes. After which the reaction was quenched by adding 150 μl stop solution (50 mM H_2SO_4).

The amount of bound conjugate can be calculated as this is proportional to the intensity of colour in each well, and therefore equivalent to the amount of RANTES present. The colour intensity was calculated using a microtitration plate reader at a wavelength of 490 nm. An average optical density ($O.D._{490}$) was calculated for each set of duplicate samples. The average of the blank wells was then subtracted from the average optical density to give a net $O.D._{490}$. Standard curves were plotted using Curve fit/cubic spline (**Fig. 2.2**). The RANTES content of each test sample was then calculated from this

standard curve.

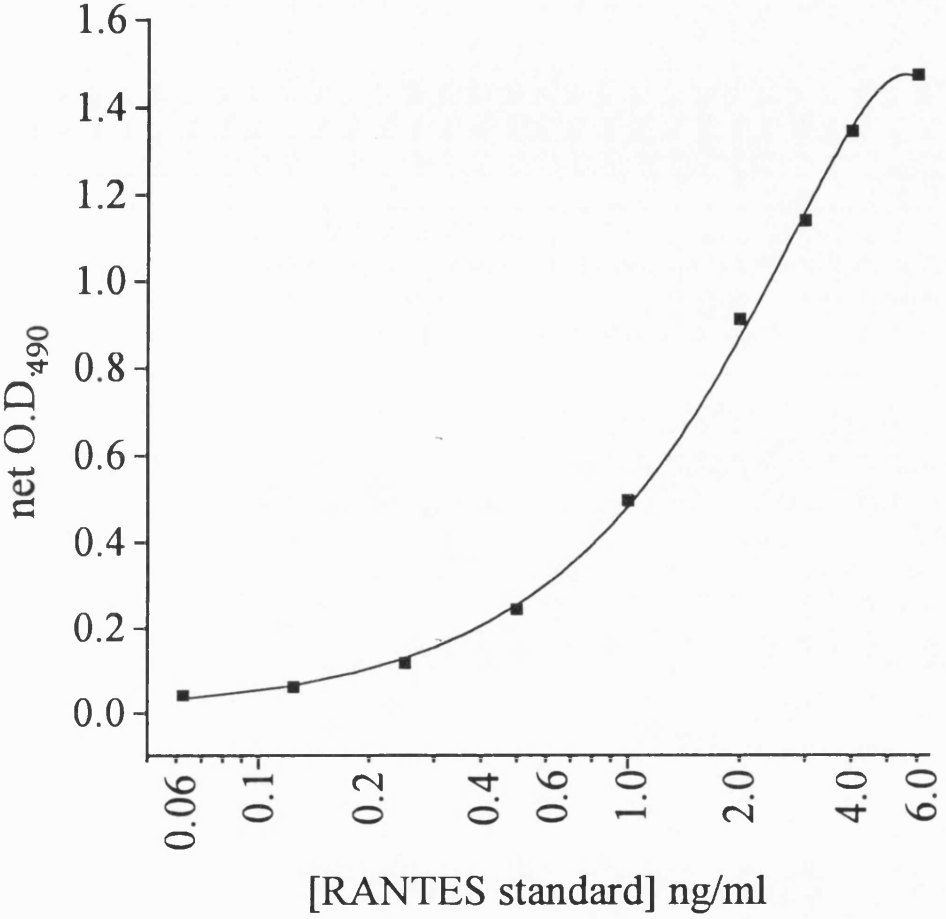


Fig. 2.2 Representative Standard Curve for RANTES ELISA

A representative standard curve for the detection of known concentrations of the RANTES peptide (6 - 0.63 ng/ml) by a double ligand sandwich ELISA. The average optical density ($O.D._{490}$) was calculated for each set of duplicate wells and the net $O.D._{490}$ was calculated by subtracting the average of the blank wells. The RANTES content of each test sample was then calculated from this standard curve.

2.13 Analysis of CD25 Expression

Another useful measure of lymphocyte function and activation is to monitor the expression of the IL-2 receptor complex (IL-2R) on the T lymphocytes after stimulation. The IL-2R is a multimeric structure of three polypeptides, a 55 kDa α-chain, a 75 kDa ß-chain and a 64 kDa γ-chain which associate to form a high affinity IL-2R. We analysed the expression of the 55 kDa α-chain which is recognised by mouse anti-human CD25 antibody.

Primary T lymphocytes were purified from PBMCs using a R+D enrichment column, as described in 2.3.2. The purified T lymphocytes were resuspended at 2 x 10^6 cells/ml in RPMI 1640 supplemented with 10 % HIBS. 470 μl T lymphocyte suspension was aliquoted into the wells of a 24-well, flat bottomed microtitre plate. The wells were treated with 10 μl vehicle, UCHT1, fixed CHO-B7$^+$, RANTES, or a combination, and made up to a final volume of 500 μl with vehicle. The cells were then incubated for 48 hours at 37 °C, 5 % CO_2.

After incubation the cells were washed twice in RPMI 1640 before incubation with either the mouse anti-human CD25 (anti-IL-2 receptor)(1:50 dilution in RPMI 1640) which was conjugated to FITC, or a control IgG antibody conjugated to FITC to check for non-specific binding, for 30 minutes at 4°C. The cells were then washed with sterile PBS twice and then analyzed on a Becton Dickinson FACS vantage (excitation wavelength - 488 nm, emission wavelength - 530 $\pm$ 15 nm).

2.14 IL-2 Production and Quantitation

The production of IL-2 by purified T lymphocytes stimulated with various treatments can be measured using the CTLL cell line which are IL-2 dependent (Ward & Cantrell, 1989; Rayter *et al.* 1992).

Primary T lymphocytes were purified from PBMCs using a R+D enrichment column. The purified T lymphocytes were resuspended at 2 x 10^6 cells/ml in RPMI 1640 supplemented with 10 % HIBS. 470 μl T lymphocyte suspension was aliquoted into the wells of a 24-well, flat bottomed microtitre plate. The wells were treated with 10 μl vehicle, UCHT1, fixed CHO-B7$^+$, RANTES, or a combination and the final volume made up to 500 μl with vehicle. The cells were then incubated for 24 hours at 37 °C, 5 % CO_2. After incubation, the cells were pelleted (450 g, 7 minutes) and the cell free supernatants were removed by suction, sub-aliquoted and stored frozen at - 20 °C.

The CTLL assay was carried out by Dr. J. McLeod. In summary, CTLL cells which had been fed with rIL-2 36 hours previously were used for the IL-2 assays. These cells were washed, then incubated in complete medium without rIL-2 for at least 1 hour. 100 μl of diluted rIL-2 standards (0.019 - 20 I.U./ml) were aliquoted into 96 well microtitre culture plates. 100 μl of assay supernatants were added to the test wells. 100 μl containing 5 x 10^4 CTLL cells were added to each well. The cells were incubated with the supernatants for 24 hours (37 °C, 5 % CO_2). 0.5 μCI [^{3}H] - thymidine was added to each well. The cells were then incubated for another 4 hours, harvested onto Whatman GF/A filters using a multiple automated cell harvester.

The incorporation of [^{3}H]-thymidine was measured using standard liquid scintillation

counting techniques. The radioactivity collected on the filter paper was assayed by placing it in 4 mls of scintillation fluid (Optiphase) and counted on a ß-scintillation counter (LKB) and expressed as cpm (Laughton *et al.* 1994).

An average cpm reading (± SEM) was calculated for each set of triplicate standards. Standard curves were plotted using a logistic (**Fig. 2.3**). The IL-2 content of each test sample was then calculated from this standard curve.

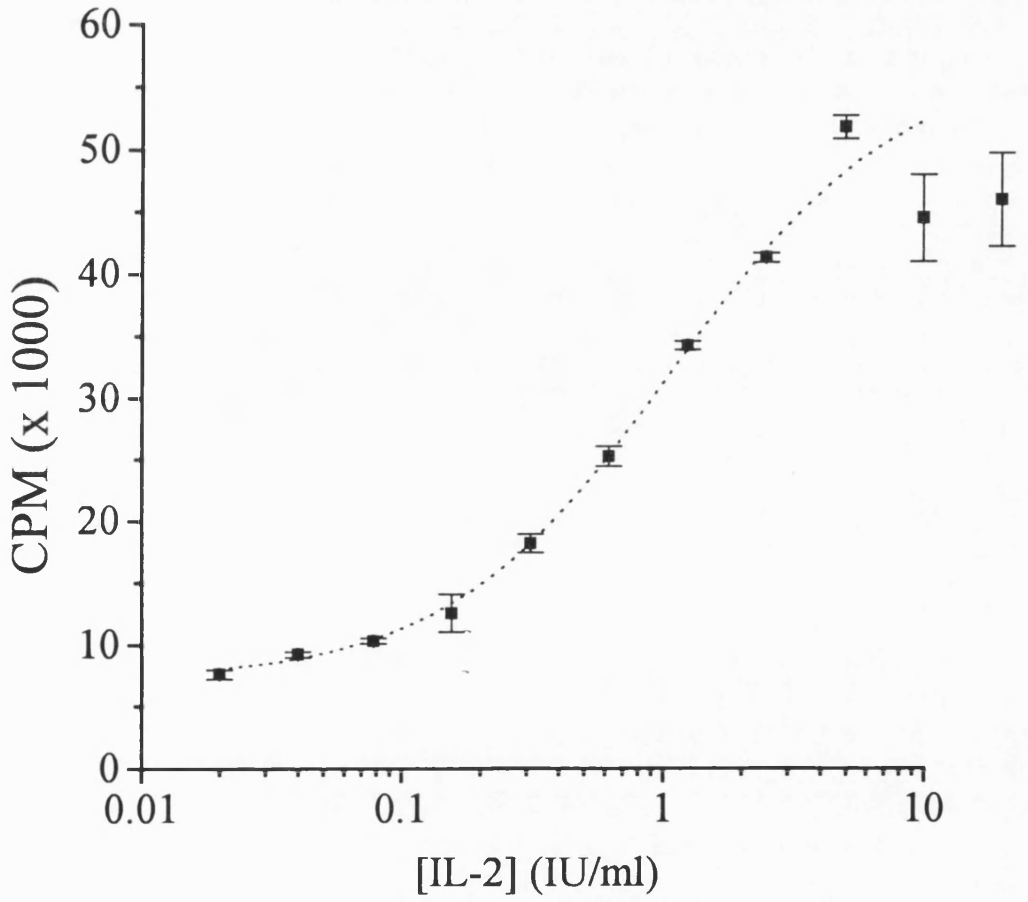


Fig. 2.3 Representative Standard Curve for IL-2 Quantitation

A representative standard curve for the detection of known concentrations of the IL-2 peptide (0.008 - 8.210 ng/ml) by a CTLL cell line assay. The IL-2 content of each test sample was then calculated from this standard curve.

Statistical Analysis

The results in the text, figures and tables of this thesis are presented as the mean ± standard error of the mean (SEM) of "n" experiments unless otherwise stated. Statistical significance was evaluated using the Student's t-test (paired or unpaired) or where appropriate, effects of treatment compared to control were analyzed by two-way analysis of variance, then Dunnett's test was used for comparison of control to treatment.

SECTION THREE

RESULTS

3.1 Induction of Migration of T Lymphocytes by RANTES

3.1.1 Purity of Primary T Lymphocyte Preparations

Primary T lymphocytes purified from PBMCs by plastic adherence and using a R+D enrichment column were stained with antibodies raised against CD3 (T cell marker), CD14 (monocytic cell marker), CD16 (NK cell marker), CD19 (B cell marker) (**Table 3.1 and Table 3.2**). The purification methods were satisfactory producing a relatively pure T lymphocyte population with very few contaminating cells. Purification by plastic adherence or using the R+D column produced a 84.2 $\pm$ 1.65 % $CD3^+$ population and 84.78 $\pm$ 4.12 % $CD3^+$ population, respectively (n = 5). The two methods of purification did not significantly affect the purity of the populations obtained when compared using a unpaired Student's t-test ($p > 0.05$).

Table 3.1 Extent of Purification of T Lymphocytes from PBMCs Using Plastic Adherence

Donor	control antibody	$CD3^+$	$CD14^+$	$CD16^+$	$CD19^+$
A	0.11	84.1	2.3	0.6	0.5
B	0.08	83.3	1.9	2.34	3.8
C	0.02	90.1	4.1	1.1	2.1
D	0.12	79.9	0.8	0.4	0.6
E	0.21	83.6	2.4	0.5	2.1

T lymphocyte enriched populations were purified from PBMCs, using plastic adherence. The % cells positive for antibodies raised against CD3, CD14, CD16, and CD19 were examined using a FACS based assay.

Table 3.2 Extent of Purification of T Lymphocytes from PBMCs Using A R+D Enrichment Column

Donor	control antibody	$CD3^+$	$CD14^+$	$CD16^+$	$CD19^+$
F	0.21	68.9	1.8	1.5	1.2
G	0.18	89.6	2.3	1.5	1.9
H	0.26	85.6	1.4	0.9	0.8
I	0.05	92.4	3.1	0.3	0.2
J	0.11	87.4	2.2	1.9	2.0

T lymphocyte enriched populations were purified from PBMCs, using a R+D enrichment column. The % cells positive for antibodies raised against CD3, CD14, CD16, and CD19 were examined using a FACS based assay.

3.1.2 Effect of RANTES on the Migration of Primary T lymphocytes

RANTES (0.1 - 100 ng/ml) stimulates locomotion of T lymphocytes, purified by plastic adherence. Peak chemotactic effects were produced with 1 or 10 ng/ml RANTES, depending on donor (**Fig. 3.1**). Low concentrations (0.1 ng/ml) of RANTES and high concentrations (100 ng/ml) did not increase migration of the T lymphocytes above vehicle control levels producing a "bell-shape-like" concentration dependent response curve. The actual number of cells migrating was largely dependent upon the cell preparations used, with T lymphocyte populations from different donors having a marked difference in response to the chemokine. Peak chemotactic effects with 1 ng/ml ranged from 40 $\pm$ 19.2 to 130 $\pm$ 32.4 cells migrating in 5 high power fields (n = 6).

Time course experiments were also carried out on the primary T lymphocytes, purified by plastic adherence, and their chemotactic response to 1 and 10 ng/ml RANTES. The locomotion was incubation time dependent (**Fig. 3.2**). A "bell-shape-like" time course response curve was produced and an incubation time of 4 hours was found to be optimal.

Checkerboard analysis was used to distinguish whether RANTES-induced migration is chemotactic or chemokinetic (**Table 3.3**). Chemokinesis is the increase of migration of random turning cells and will occur when there is no concentration gradient present, i.e. when there are equal concentrations of chemoattractant in the upper and lower wells. Chemotaxis, however, is the vectorial migration of cells along a concentration gradient. Migration of primary T lymphocytes was induced significantly, with higher concentrations of RANTES in the lower wells. There was little migration when the concentration of RANTES in the upper and lower wells was equal. Hence, the primary

T lymphocyte response to RANTES is a chemotactic response rather than the result of an increase in random migration.

Table 3.3 Checkerboard Analysis of RANTES-Induced Migration of Primary T Lymphocytes

cells migrating in five high power fields				
upper well→ lower well↓	0 ng/ml	0.1 ng/ml	10 ng/ml	1000 ng/ml
0 ng/ml	9 (±1.0)	21.6 (±6.5)	18.5 (±6.9)	5 (±2.0)
0.1 ng/ml	31 (±15.8)	16.3 (±11.3)	32.3 (±14.5)	14.5 (±0.5)
10 ng/ml	93.3 (±9.2)	60.3 (±5.3)	28.6 (±7.5)	27 (±5.0)
1000 ng/ml	24.6 (±10.5)	54.6 (±8.9)	36.3 (±6.8)	32 (±8.3)

Purified T lymphocytes were assayed for migration in response to RANTES, with the indicated concentration of RANTES (0.1 - 1000 ng/ml) in the upper and lower wells, shown above and to the left of the matrix, respectively. Each value is mean (± SEM) of triplicate or quadruplicate wells in a single representative experiment.

3.1.3 Effect of RANTES on Migration of Jurkat T Cells

RANTES (0.1 - 100 ng/ml) failed to cause migration of Jurkat T cells above vehicle control levels (**Fig. 3.3 a)**). The incubation time with RANTES was not a factor as incubation with 1 ng/ml or 10 ng/ml RANTES over a time course of 1 - 5 hours did not lead to increased migration of the Jurkat T cells above vehicle control levels (**Fig. 3.3 b)**).

Several microchemotaxis chamber polycarbonate membranes were used with pore sizes of 5, 8, and 12 μm pore size but RANTES-induced migration was still not observed above basal levels (n = 2, for each pore size).

3.1.4 Effect of RANTES on Migration of Primary T Lymphocytes Purified from Blood Packs

T lymphocytes that had been purified from whole blood packs by plastic adherence, obtained from the Blood Transfusion Service, were also used in chemotaxis experiments. RANTES (0.1 - 100 ng/ml) did not induce migration of these T lymphocytes (**Fig. 3.4 a)**). Altering the incubation time with RANTES, over a time course of 1 - 5 hours, did not cause induction of migration either (**Fig. 3.4 b)**). The T lymphocytes were purified from 4 day old blood that had been donated to the Blood Transfusion Service.

3.1.5 Effect of RANTES on Migration of Quiescent T Lymphoblasts

RANTES (0.1 - 100 ng/ml) did not induce a chemotactic response in quiescent T lymphoblasts, 10 - 14 days old, above that of vehicle control levels (**Fig. 3.5 a)**). The incubation time with RANTES was not a factor as incubation with 1 ng/ml or 10 ng/ml RANTES over a time course of 1 - 5 hours did not lead to increased migration of the quiescent T lymphoblasts above vehicle control levels (**Fig. 3.5 b)**). Different polycarbonate membrane with varying pore sizes (5 - 10 μm) were used in the 48-well microchemotaxis chamber but RANTES still did not induce migration of the quiescent T lymphoblasts.

3.1.6 Effect of RANTES on Migration of T Lymphoblasts

The effect of RANTES on *in vitro* migration of T lymphoblasts was assessed at several stages in their preparation from PBMC : after stimulation with PHA for 3 days (**Fig. 3.6 a)**), after stimulation with PHA for 3 days followed by incubation with IL-2 for 48 hours (**Fig. 3.6 b)**), after stimulation with PHA for 3 days followed by incubation with IL-2 for 4 days (**Fig. 3.6 c)**), after stimulation with PHA for 3 days followed by incubation with IL-2 for 10 days (**Fig. 3.6 d)**). RANTES (0.1 - 100 ng/ml) did not induce migration of any of the cell preparations after incubation for 4 hours in a 48-well microchemotaxis chamber above the levels of migration seen with vehicle control only.

3.1.7 Comparison of RANTES Induced Migration in Primary T Lymphocytes Purified by Two Different Methods

Primary T lymphocyte were purified from the same blood donations by plastic adherence and using the R+D T cell enrichment column. The cells were then incubated with RANTES (0.1 - 100 ng/ml) in a microchemotaxis assay for 4 hours (*n* = *3*)(**Fig. 3.7**). The different methods of purification did not alter RANTES-induced migration.

3.1.8 Induction of Polarisation of T Lymphocytes by RANTES

RANTES induced polarisation of T lymphocytes, recognised by a change in shape from spherical lymphocytes to a polarised, elongated form (**Fig. 3.8**). RANTES (0.1 - 1000 ng/ml) induced an increase in polarisation of primary T lymphocytes, purified by plastic adherence. A "bell-shape-like" concentration dependent response curve was produced and peak polarisation was achieved in response to 1 to 10 ng/ml RANTES (**Fig. 3.9**). Although the absolute number of cells that responded to RANTES was dependent upon the donor used, the fold increase in polarised cells compared with vehicle-treated cells was consistent for each RANTES concentration tested. For example, cells polarised with RANTES (10 ng/ml) induced a significant increase in polarisation ($p < 0.01$, two way analysis of variance), with a 4.56 ± 0.42 - fold increase in percentage of cells polarised, compared with vehicle-treated cells ($n = 5$)(**Table 3.4**).

RANTES-induced polarisation of primary T lymphocytes was time dependent. Experiments with cells treated with 1 ng/ml RANTES over a time course of 30 - 240 minutes showed that RANTES polarisation had optimal effects within 60 minutes (**Fig. 3.10**).

Table 3.4 Polarisation in Response to RANTES

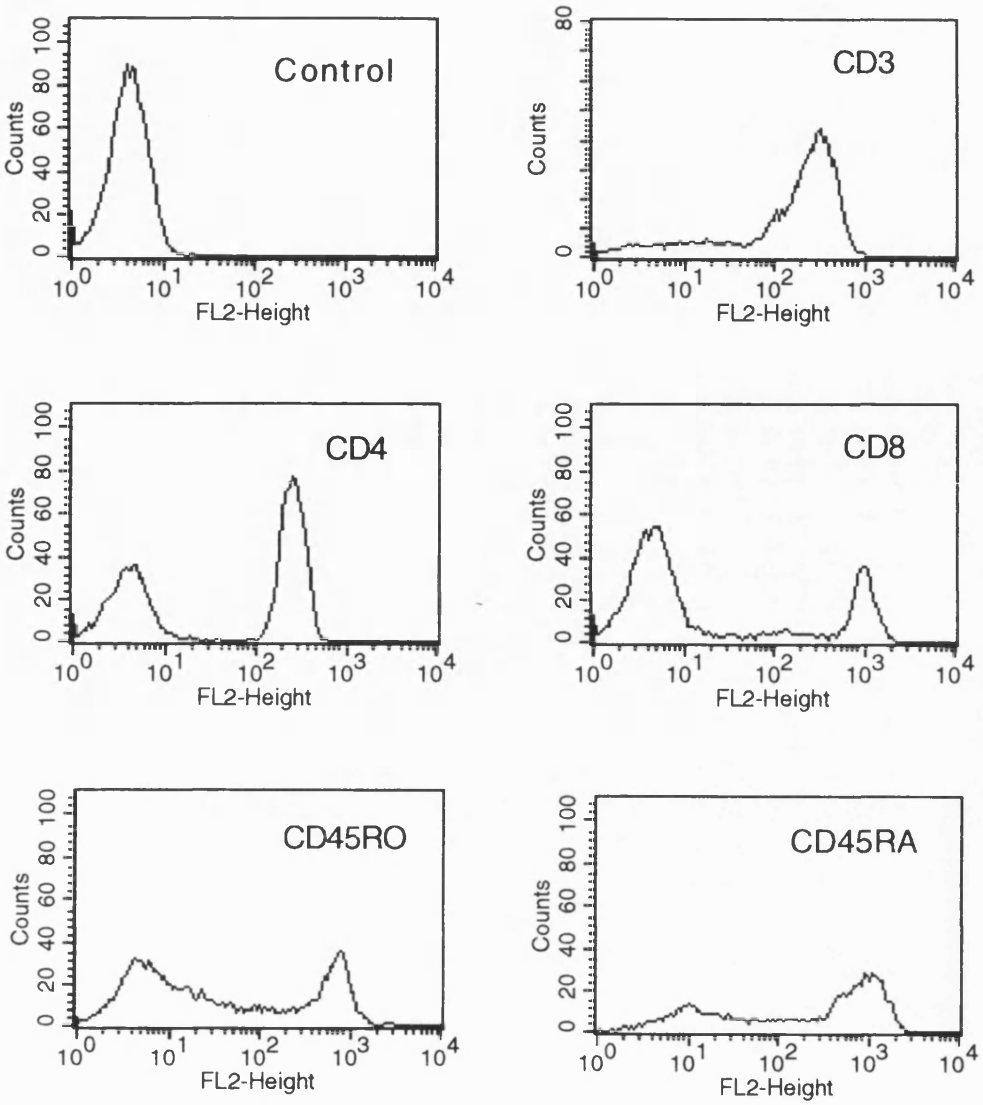
Donor	% cells polarised in response to vehicle	% cells polarised in response to 10 ng/ml RANTES	fold increase in polarisation
1	1.2 ± 0.8	5.3 ± 1.6	4.41
2	6.1 ± 2.6	27.2 ± 4.2	4.46
3	4.2 ± 0.6	16.1 ± 2.1	3.83
4	3.4 ± 1.2	12.9 ± 2.9	3.79
5	2.8 ± 0.3	13.1 ± 3.1	4.68

T lymphocytes were purified using plastic adherence then incubated with vehicle control or 10 ng/ml RANTES for 1 hour. After that time the extent of polarisation was assessed.

3.1.9 Comparison of RANTES Induced Polarisation in Primary T Lymphocytes Purified By Two Different Methods

Primary T lymphocyte were purified from the same blood donations by plastic adherence and using the R+D T cell enrichment column. The cells were then incubated with RANTES (0.1 - 100 ng/ml) for 1 hour (n = 3)(**Fig. 3.11**). The extent of polarisation induced by RANTES in the cells purified by the different methods was compared. RANTES-induced polarisation was not affected by the purification method used.

In addition, polarisation assays were performed on different primary T lymphocyte populations and the extent of RANTES (1 ng/ml) induction of polarisation was examined. In addition, the T cell subsets present in these populations were examined using antibodies against CD4, CD8, CD45RO and CD45RA cell surface antigens. The extent of RANTES (1 ng/ml) induction of polarisation in the different populations was not related to differences in the T cell subsets present (**Table 3.5**).



FACS Traces of staining with amtibodies against T lymphocyte antigens.

Table 3.5 Surface Expression of T Lymphocyte Subset Markers

Donor	control antibody	$CD3^+$	$CD4^+$	$CD8^+$	$CD45RA^+$	$CD45RO^+$	% cells polarised in response to RANTES
6	0.24	86.3	37.7	33.7	29.1	48.9	15.43
7	0.34	91.6	43.2	39.8	21.3	31.6	10.78
8	0.02	92.1	37.3	42.5	61.0	73.4	8.95
9	0.18	79.4	38.8	36.4	35.4	61.2	13.65
10	0.68	82.3	39.4	40.8	29.9	34.5	14.2

T lymphocytes were purified using plastic adherence then the % cells positive for antibodies raised against CD3, CD4, CD8, CD45RA, CD45RO were examined using a FACS based assay. Each cell preparation was also incubated for 1 hour with 1 ng/ml RANTES and after that time the extent of polarisation was assessed.

3.1.10 Induction of Actin Polymerisation in T Lymphocytes by RANTES

RANTES (0.1 - 100 ng/ml) induces actin polymerisation from G-actin to F-actin in primary T lymphocytes. A "bell-shape-like" concentration dependent response curve is observed, with 10 ng/ml causing peak actin polymerisation in the cells. The optimal RANTES concentrations cause the same degree of induction of actin polymerisation induced by soluble mouse anti-human CD3 (UCHT1, 10 μg/ml) (**Fig. 3.12**).

The effects of RANTES on actin polymerisation are also time dependent. Primary T lymphocytes were incubated with 1 ng/ml RANTES over a time course of 1 - 60 minutes. RANTES induces optimal actin polymerisation within 10 minutes (**Fig. 3.13**).

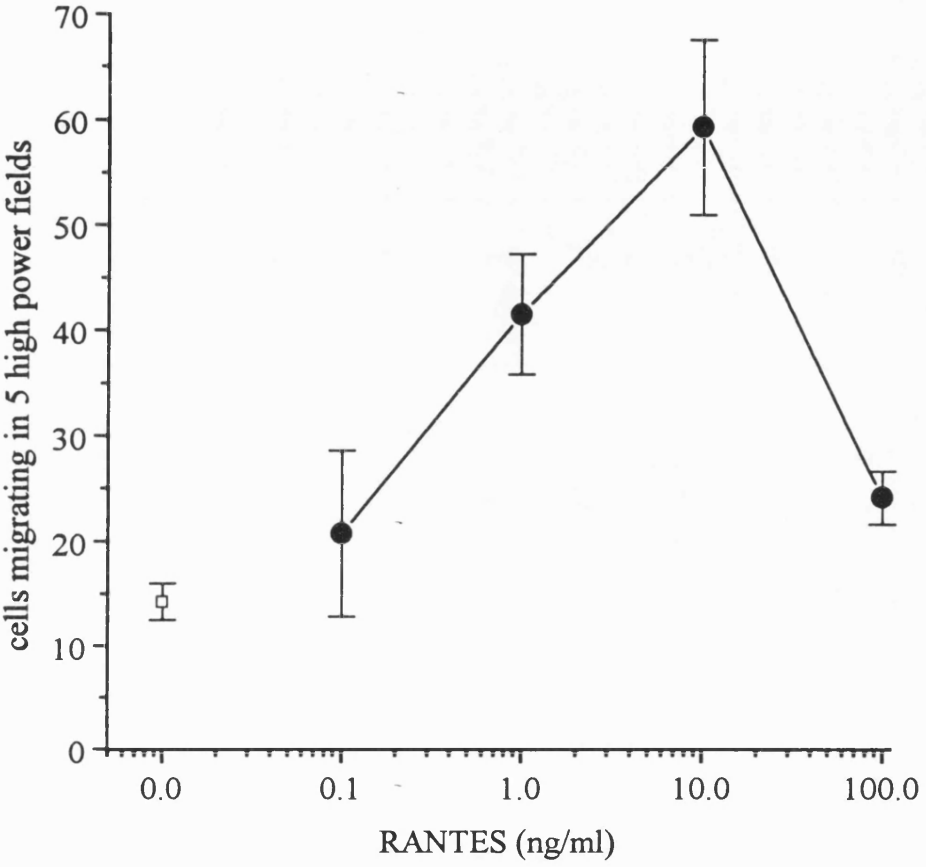


Fig. 3.1 Effect of RANTES on *In Vitro* Migration of Primary T Lymphocytes - dose response curve

The effect of vehicle control (□) and RANTES (●) on *in vitro* migration of primary T lymphocytes. The cells were purified using plastic adherence then incubated with RANTES (0.1 - 100 ng/ml) in a 48-well microchemotaxis chamber for 4 hours, as described in Materials and Methods. Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

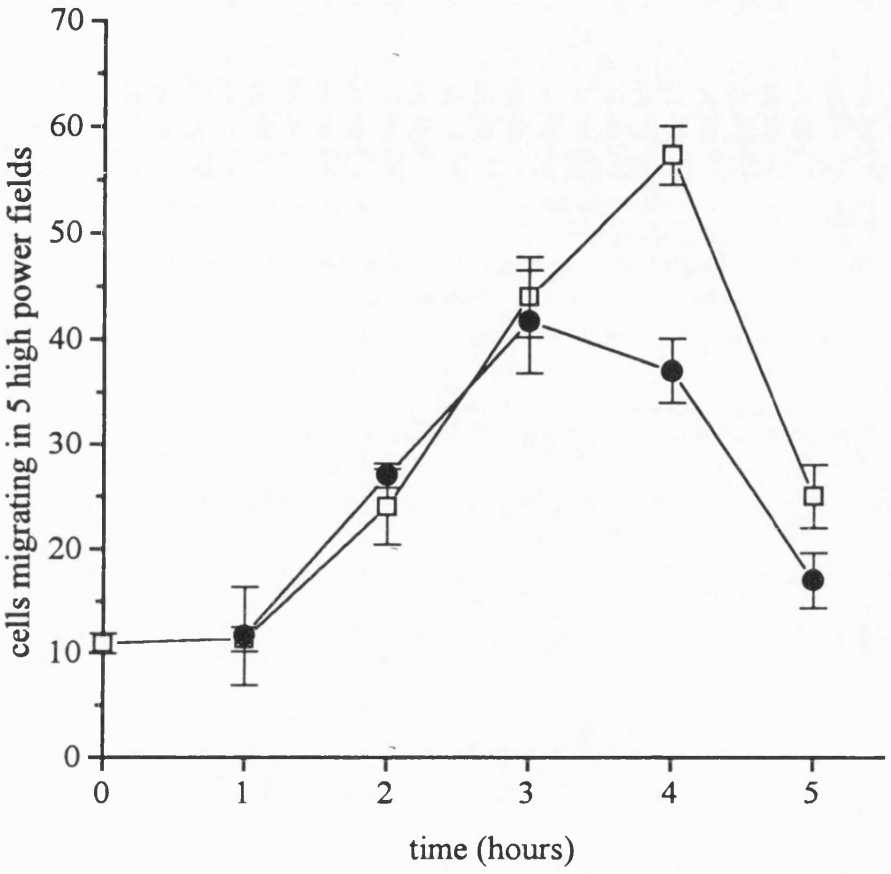


Fig. 3.2 Effect of RANTES on *In Vitro* Migration of Primary T Lymphocytes - time course

Primary T lymphocytes, purified by plastic adherence, were incubated with 1 ng/ml RANTES (□) and 10 ng/ml RANTES (●) over a time course of 1 - 5 hours in a 48-well microchemotaxis chamber. Primary T lymphocytes were incubated with vehicle controls for 5 hours. Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

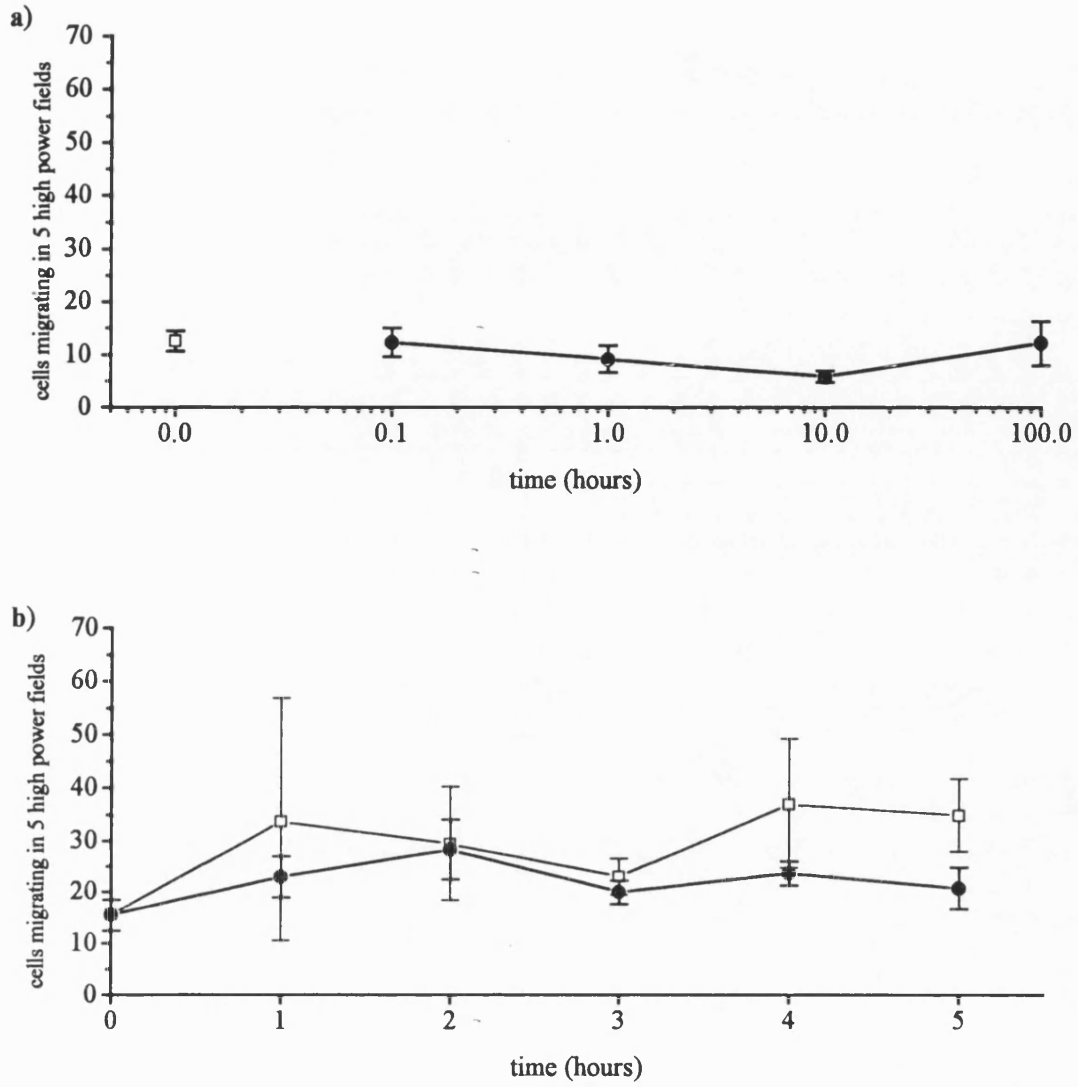


Fig. 3.3 Effect of RANTES on *In Vitro* Migration of Jurkat T Cells

a) The effect of RANTES (0.1 - 100 ng/ml, ●) and vehicle control (□) on *in vitro* migration of Jurkat T cells. The cells were incubated for 4 hours in a 48-well microchemotaxis chamber and migration was assessed as described in Materials and Methods.

b) Jurkat T cells were incubated with 1 ng/ml RANTES (□) and 10 ng/ml RANTES (●) over a time course of 1 - 5 hours in a 48-well microchemotaxis chamber. Jurkat T cells were incubated with vehicle controls for 5 hours.

Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

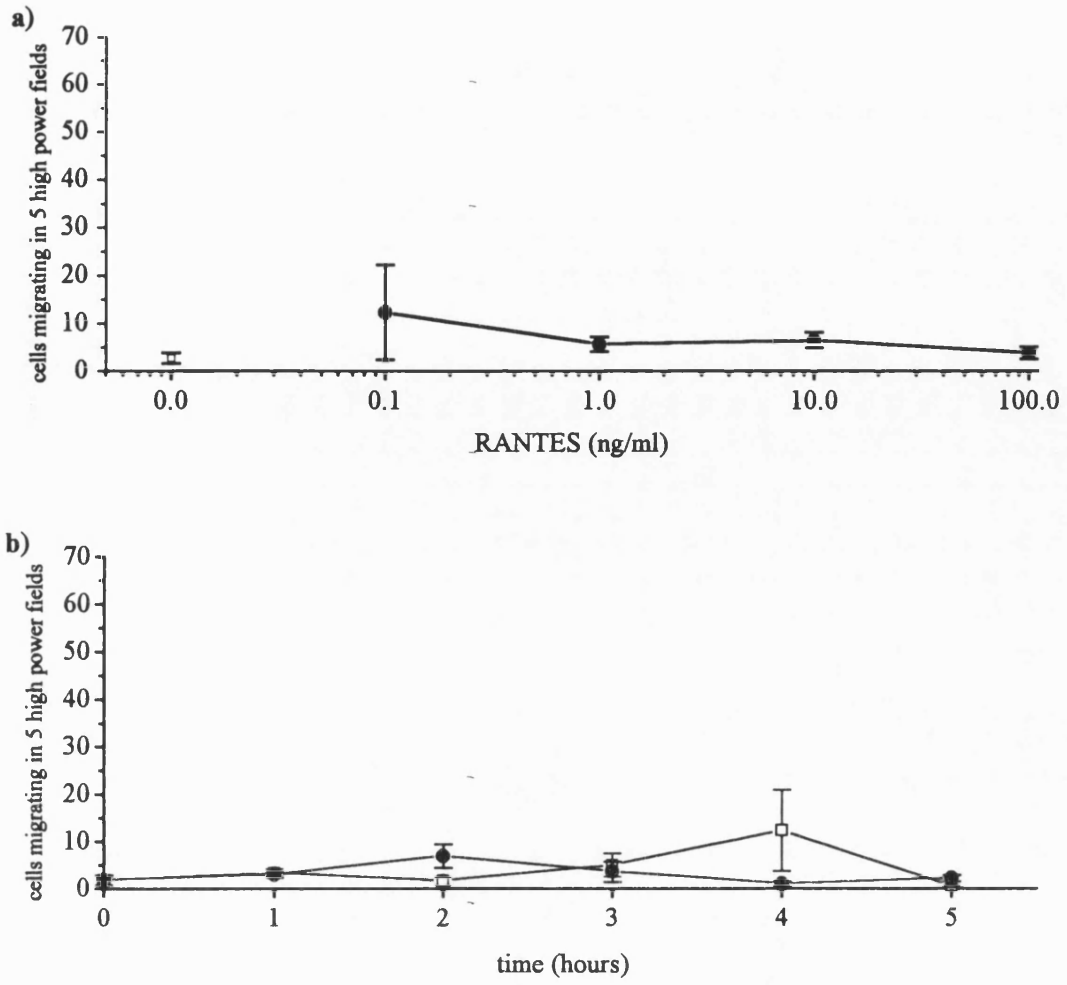


Fig. 3.4 Effect of RANTES on *In Vitro* Migration of Primary T Lymphocytes Purified From Whole Blood Packs

a) The effect of RANTES (0.1 - 100 ng/ml, ●) and vehicle control (□) on *in vitro* migration of primary T lymphocytes purified from whole blood packs. The cells were incubated for 4 hours in a 48-well microchemotaxis chamber and migration was assessed as described in Materials and Methods.

b) Primary T lymphocytes, purified from whole blood packs by plastic adherence, were incubated with 1 ng/ml RANTES (□) and 10 ng/ml RANTES (●) over a time course of 1 - 5 hours in a 48-well microchemotaxis chamber. The cells were incubated with vehicle controls for 5 hours.

Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

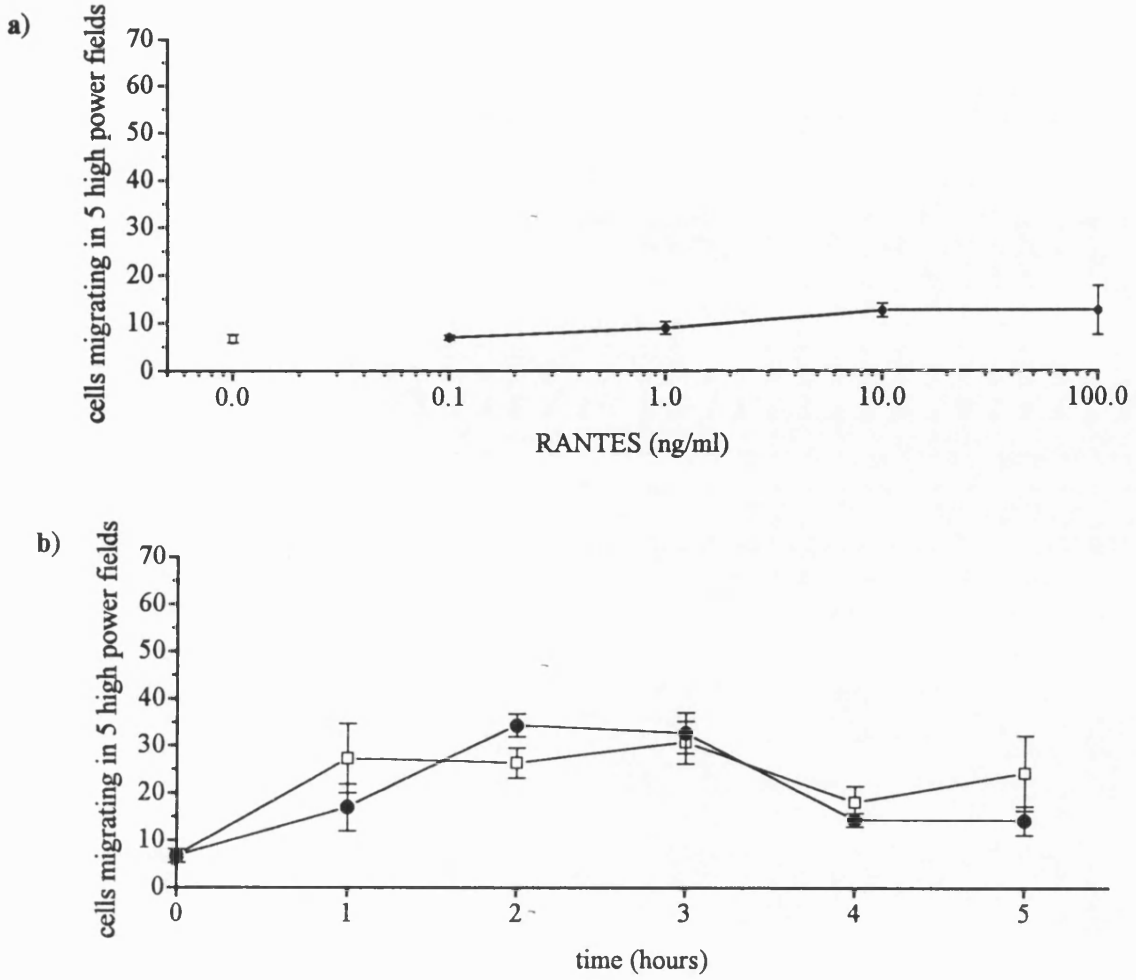


Fig. 3.5 Effect of RANTES on *In Vitro* Migration of Quiescent T Lymphoblasts

a) The effect of RANTES (0.1 - 100 ng/ml, ●) and vehicle control (□) on *in vitro* migration of quiescent T lymphoblasts. The cells were incubated for 4 hours in a 48-well microchemotaxis chamber and migration was assessed as described in Materials and Methods.

b) Quiescent T lymphoblasts were incubated with 1 ng/ml RANTES (□) and 10 ng/ml RANTES (●) over a time course of 1 - 5 hours in a 48-well microchemotaxis chamber. The cells were incubated with vehicle controls for 5 hours.

Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

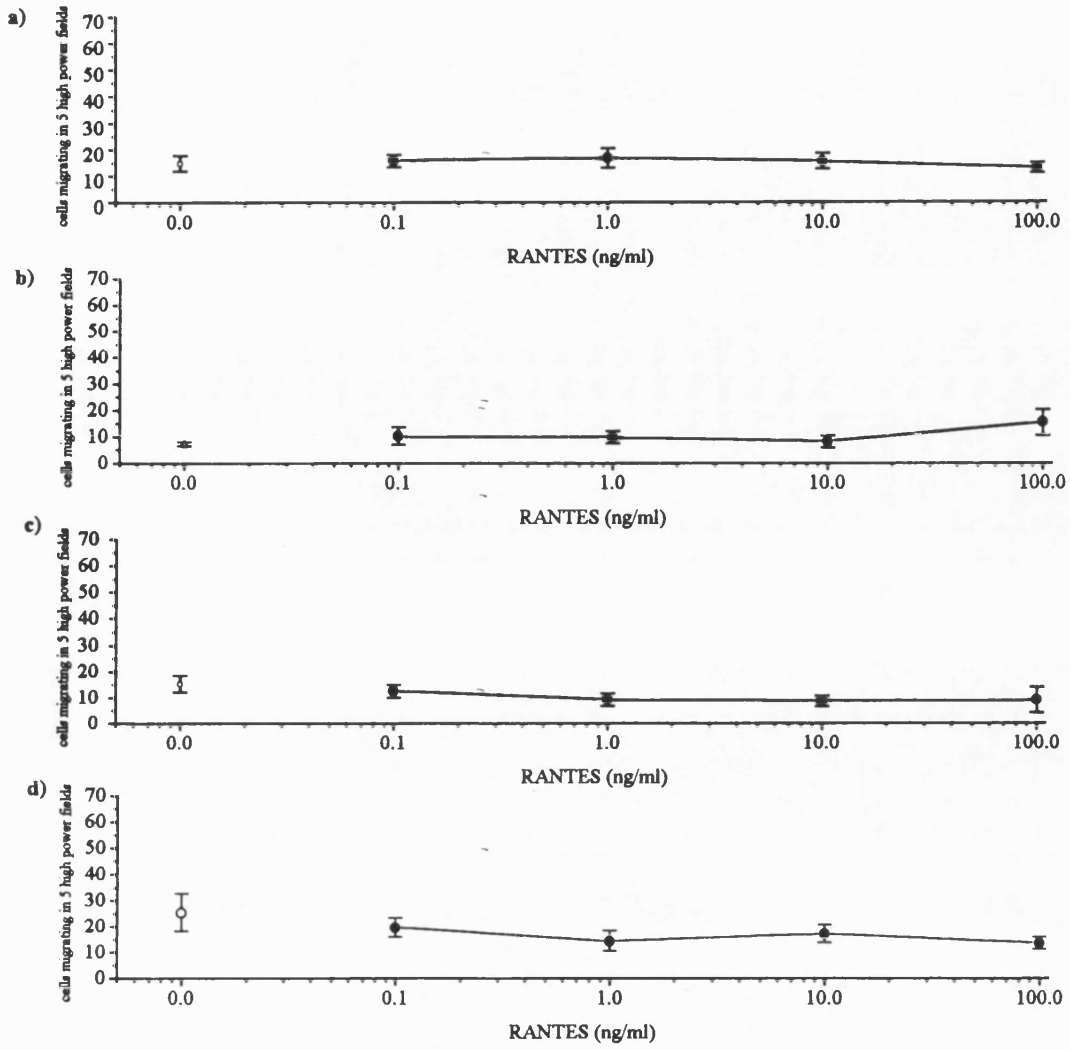


Fig. 3.6 Effect of RANTES on *In Vitro* Migration of PBMC Stimulated to Produce T Lymphoblasts

The effect of RANTES (0.1 - 100 ng/ml, ●) and vehicle control (□) on *in vitro* migration of a)PBMC which had been stimulated for three days with PHA. b)PBMC which had been stimulated for 72 hours with PHA, followed by incubation with IL-2 for 48 hours. c)PBMC which had been stimulated for three days with PHA, followed by incubation with IL-2 for four days. d)PBMC which had been stimulated for 72 hours with PHA, followed by incubation with IL-2 for 10 days.

The cells were washed then incubated for 4 hours in a 48-well microchemotaxis chamber and migration was assessed as described in Materials and Methods. Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

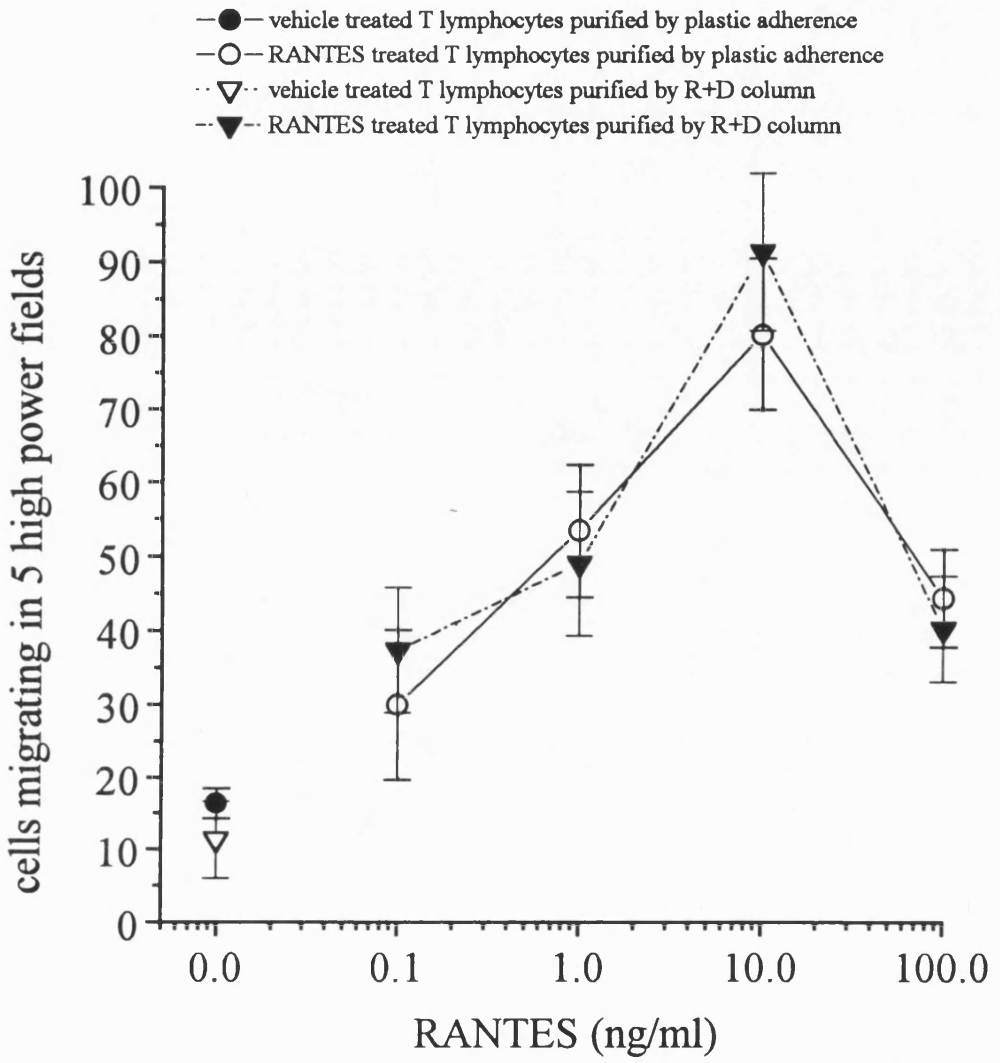


Fig. 3.7 Comparison of RANTES Induced Migration of Primary T Lymphocytes Purified by Two Different Methods

The effect of vehicle control (□) and RANTES (●) on *in vitro* migration of primary T lymphocytes. The cells were purified by the two different methods, plastic adherence and using a R+D enrichment column. The cells were then incubated with RANTES (0.1 - 100 ng/ml) in a 48-well microchemotaxis chamber for 4 hours, as described in Materials and Methods. Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least two others performed.

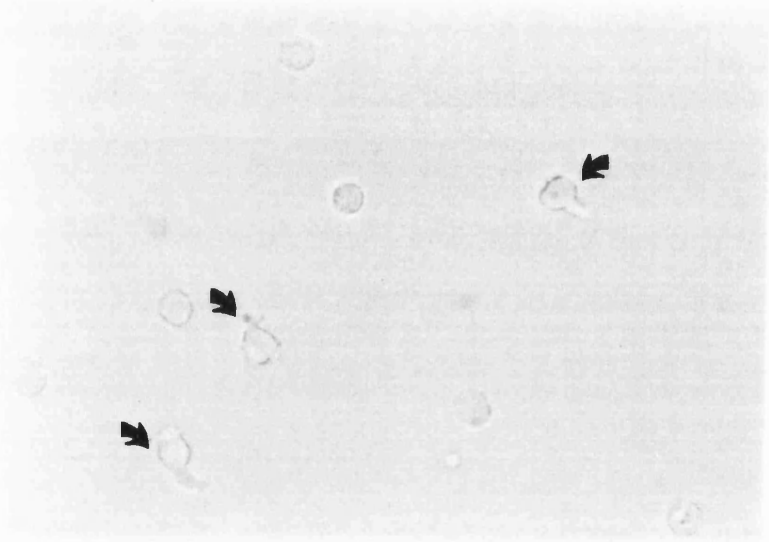


Fig 3.8 RANTES Induced Polarisation of Primary T Lymphocytes

Example of RANTES induced polarisation of primary T lymphocytes. RANTES induced T lymphocyte polarisation is indicated by ➚ .

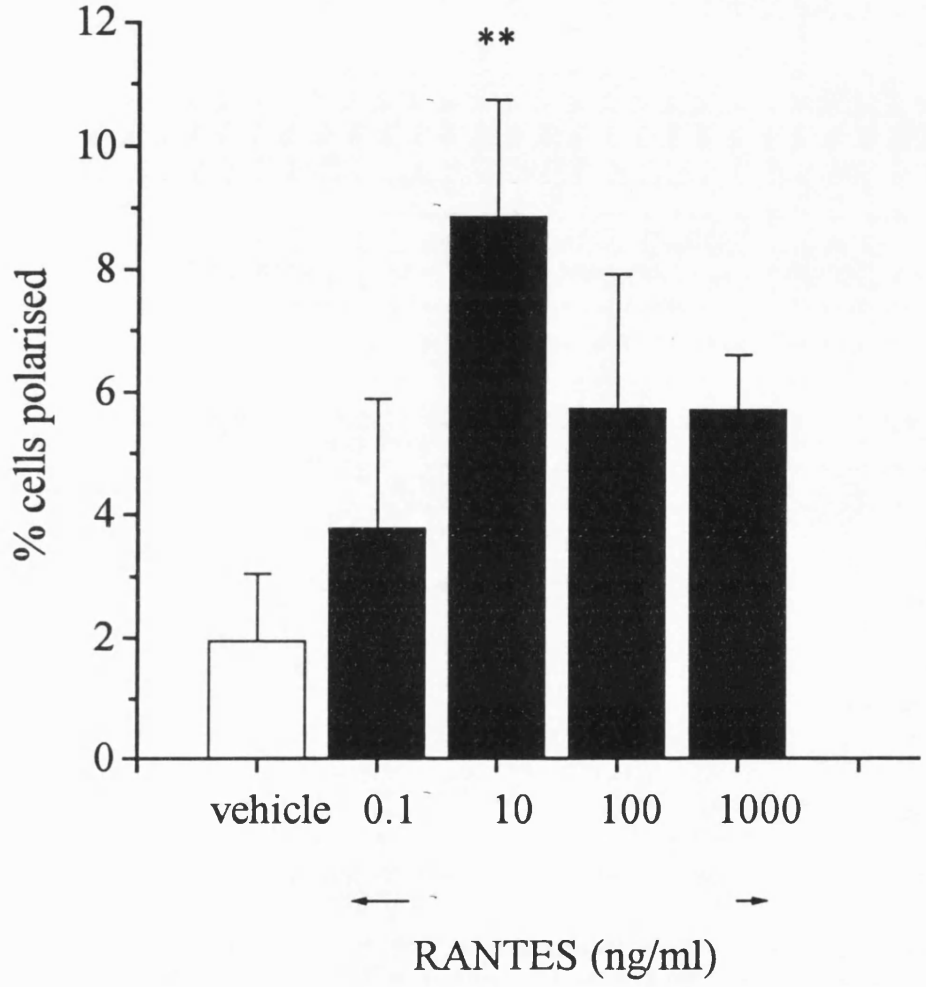


Fig 3.9 RANTES Induced Polarisation of Primary T Lymphocytes - dose response curve

Polarisation of T lymphocytes, purified by plastic adherence, treated with RANTES (0.1 - 1000 ng/ml, ●) or vehicle control (□). The cells were incubated with RANTES or vehicle control for 60 minutes. Results are expressed as the proportion of T lymphocytes that polarise after culture with RANTES. Each treatment was repeated in triplicate and each point is the mean (± SEM) of these triplicates. Results are from a single representative experiment of three others performed.

Significant at ** $p < 0.01$, two way analysis of variance.

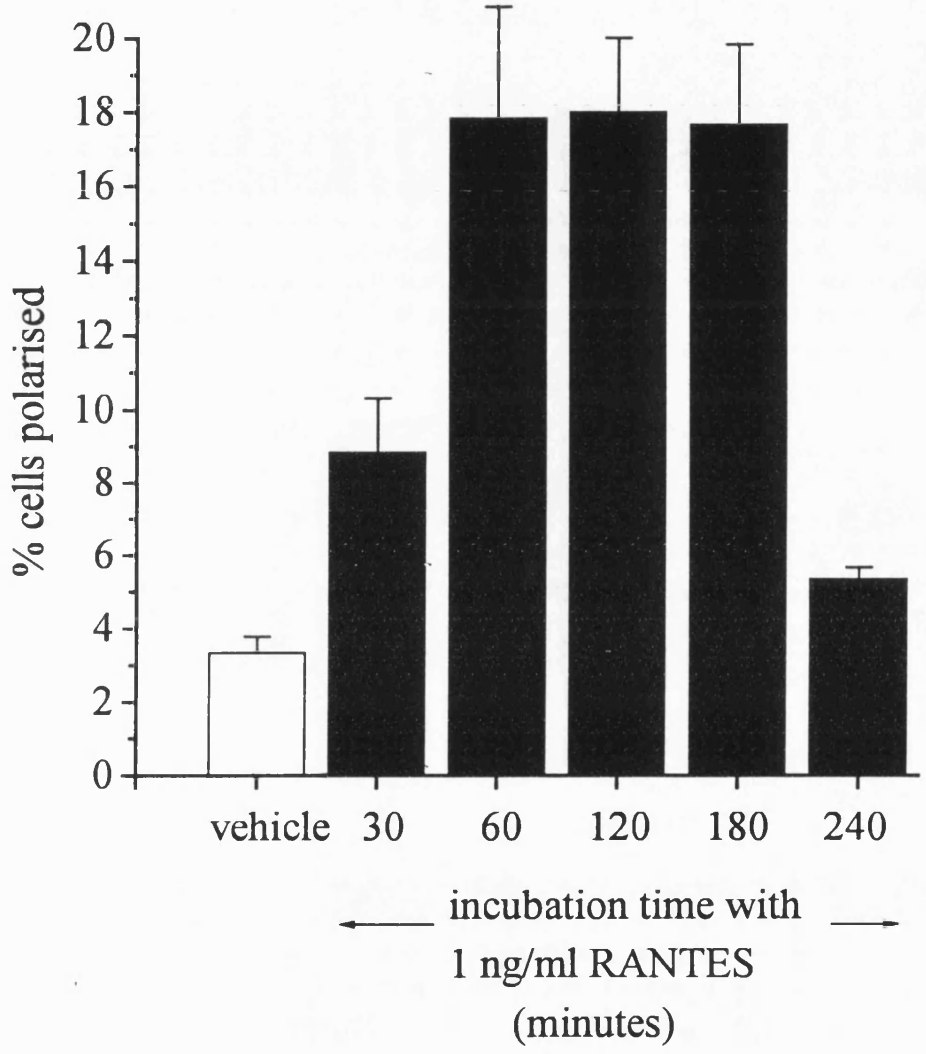


Fig. 3.10 RANTES Induced Polarisation of Primary T Lymphocytes - time course

Polarisation of T lymphocytes, purified by plastic adherence, treated with 1 ng/ml RANTES over a time course of 30 - 240 minutes (■). T lymphocytes were incubated with vehicle control for 240 minutes (□). Results are expressed as the proportion of T lymphocytes that polarise after culture with RANTES. Each treatment was repeated in triplicate and each point is the mean (± SEM) of these triplicates. Results are from a single representative experiment of three others performed.

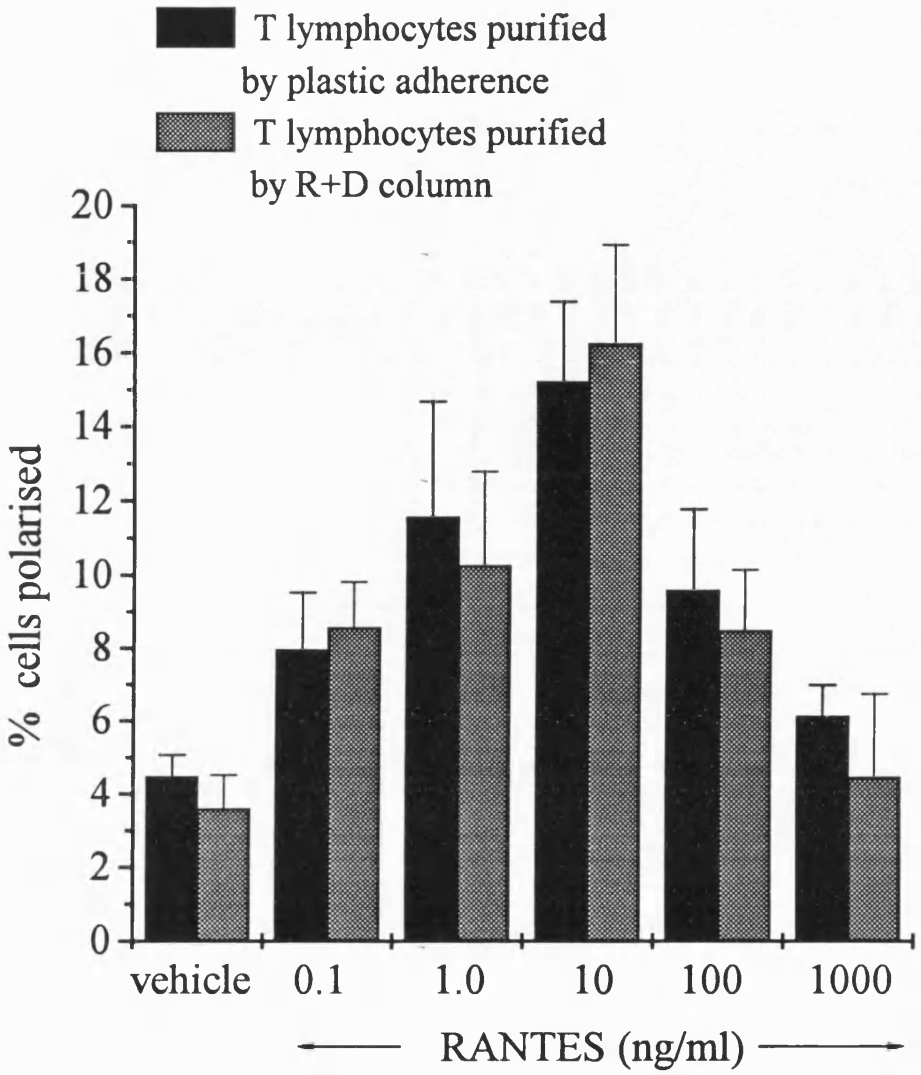


Fig 3.11 RANTES Induced Polarisation of Primary T Lymphocytes - comparison of purification methods

Polarisation of T lymphocytes, purified by plastic adherence or purified using a R + D enrichment column, treated with RANTES (0.1 - 1000 ng/ml) or vehicle control. The cells were incubated with RANTES or vehicle control for 60 minutes. Results are expressed as the proportion of T lymphocytes that polarise after culture with RANTES. Each treatment was repeated in triplicate and each point is the mean (± SEM) of these triplicates. Results are from a single representative experiment of two others performed.

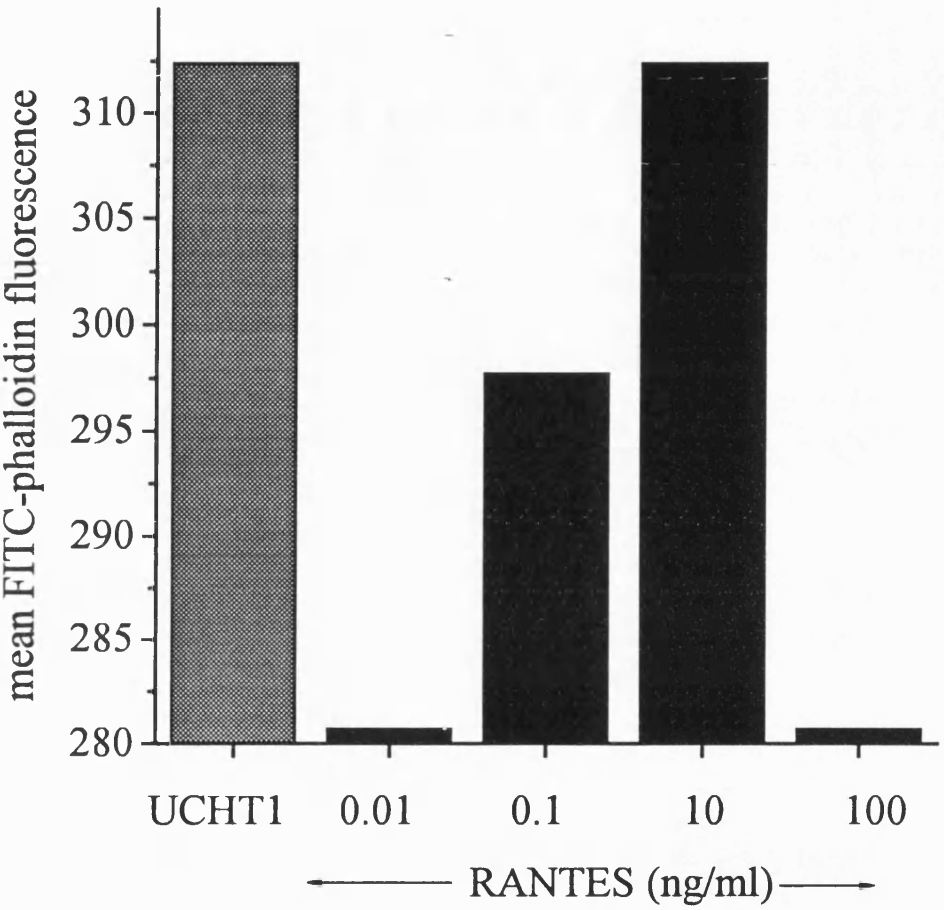


Fig. 3.12 RANTES Induced Actin Polymerisation of Primary T Lymphocytes - dose response curve

T lymphocytes, purified by plastic adherence, were treated with RANTES (0.01 - 100 ng/ml, ■) or UCHT1 (10 μg/ml, ▩). The cells were incubated with RANTES or UCHT1 for 10 minutes, fixed, then the degree of actin polymerisation was assessed using FITC-phalloidin, which only binds to F-actin, not the unpolymerised form, see Materials and Methods. Results are from a single representative experiment of three others performed.

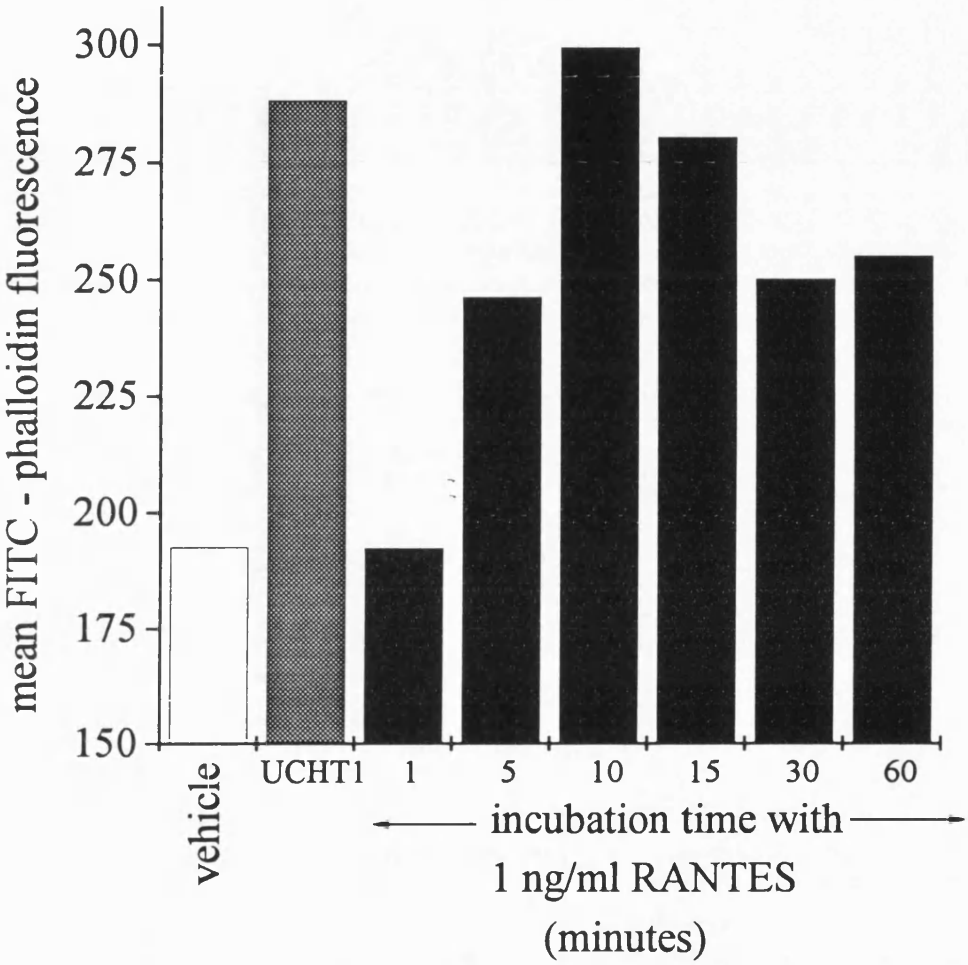


Fig. 3.13 RANTES Induced Actin Polymerisation of Primary T Lymphocytes - time course

T lymphocytes, purified by plastic adherence, were treated with 1 ng/ml RANTES over a time course of 1 - 60 minutes (■). Cells were also treated with UCHT1 (10 μg/ml, 10 minutes, ▩) and vehicle control for 10 minutes (□). The cells were fixed, then the degree of actin polymerisation was assessed using FITC-phalloidin, which only binds to F-actin, not the unpolymerised form, see Materials and Methods. Results are from a single representative experiment of three others performed.

3.2 RANTES Activated Signalling Pathways

3.2.1 Effect of RANTES on T Lymphocyte Cytosolic Calcium Concentrations

Addition of soluble mouse anti - human CD3 (UCHT1, 10 μg/ml) to fura-2 loaded Jurkat T cells significantly elevated ($p < 0.01$, two way analysis of variance) the basal $[Ca^{2+}]_i$ from a value of 38.5 $\pm$ 20.2 nM to 120 $\pm$ 18.5 nM ($n = 4$)(**Fig. 3.14 a), b)**). A signification elevation from a basal $[Ca^{2+}]_i$ of 36.3 $\pm$ 14.1 nM to 370 $\pm$ 40.2 nM ($n = 4$) was also induced by in fura-2 loaded quiescent T lymphoblasts (**Fig. 3.15 a), b)**). Elevation of $[Ca^{2+}]_i$ levels were detected 50 seconds after UCHT1 treatment and the maximal increase was seen after 200 seconds. Addition of RANTES (10 ng/ml) did not significantly elevate basal $[Ca^{2+}]_i$ in either Jurkat T cells (**Fig. 3.14 c)**) or quiescent T lymphoblasts (**Fig. 3.15 c)**)($n = 4$).

UCHT1 (10 μg/ml) caused an increase in $[Ca^{2+}]_i$ in primary T lymphocytes, purified by plastic adherence. Addition of UCHT1 significantly elevated ($p < 0.01$, two way analysis of variance) the basal $[Ca^{2+}]_i$ from a value of 32.5 $\pm$3.23 nM to 112 $\pm$ 3.46 nM ($n = 4$). with a maximal rise after 200 seconds (**Fig. 3.16**). In marked contrast, RANTES (0.1 - 100 ng/ml) during a 500 second observation period produced an elevation of 5.5 $\pm$ 1.66 nM ($n = 4$)(**Fig. 3.17**), which was not a significant difference from vehicle-treated cells. With the same preparations of lymphocytes, however, RANTES did induce migration (data not shown).

Thus we were unable to detect RANTES-induced changes in $[Ca^{2+}]_i$ in any human T lymphocyte preparation.

3.2.2 Effect of RANTES on Phosphatidylinositol Metabolism

T lymphocytes were metabolically labelled with [^{32}P]-orthophosphate and stimulated with UCHT1 or RANTES. Phosphatidylinositol metabolism was assessed by examining [^{32}P] - PtdOH production and PtdIns(4,5,)P_2 breakdown, both indicators of PLC activation, as PtdOH acid is a breakdown product of diacylglycerol. The production of [^{32}P] - labelled D-3 phosphoinositides was examined to investigate whether PI 3-kinase acitivty was induced. The results for [^{32}P] - PtdOH production and PI(4,5)P_2 breakdown are expressed as a percentage of [^{32}P] - PtdIns to control for preparation variation as [^{32}P] - PtdIns levels should remain constant. The primary T lymphocyte [^{32}P] - PtdIns results were in the range of 0.8 - 1.7 * 10^4 cpm, much lower than the quiescent T lymphoblasts, which had levels of 8 - 20 * 10^4 cpm, suggesting that the primary T lymphocytes did not incorporate the [^{32}P]-orthophosphate label adequately.

Addition of UCHT1 (10 μg/ml, 10 minute incubation) to **a)** quiescent T lymphoblasts and **b)** primary T lymphocytes, metabolically labelled with [^{32}P]-orthophosphate, increased [^{32}P] - phosphatidic acid (PtdOH) production in all preparations tested (**Fig. 3.18**)(n = 3). The fold increase in [^{32}P] - PtdOH production was 6.02 $\pm$ 2.3 for quiescent T lymphoblasts (n = 3) and 5.23 $\pm$ 3.6 for primary T lymphocytes (n = 4). RANTES (1 ng/ml 30 - 600 second incubation) did not cause any detectable changes in [^{32}P] - PtdOH production in quiescent T lymphoblasts or primary T lymphocytes (n = 3).

[^{32}P] - PtdIns(4,5)P_2 levels were decreased following UCHT1 stimulation (10 μg/ml, 10 minute incubation) in both **a)** quiescent T lymphoblasts and **b)** primary T lymphocytes, metabolically labelled with [^{32}P]-orthophosphate (n = 3)(**Fig. 3.19**). [^{32}P] - PtdIns(4,5)P_2 breakdown was detected in all cells stimulated with UCHT1, inducing a fold decrease of [^{32}P] - PtdIns(4,5)P_2 levels by 2.3 $\pm$ 0.9 in quiescent T lymphoblasts (n = 3) and 3.2 $\pm$ 1.2 in primary T lymphocytes (n = 3). RANTES (1 ng/ml, 30 - 600 second incubation) did not reduce the levels below vehicle controls.

D-3 phosphoinositides are minor fractions of the total phosphatidylinositol lipids so the D-3 phosphoinositide production results are not expressed as a fraction of the PtdIns as the percentages would be small and changes difficult to detect, instead these results are expressed in cpm. UCHT1 stimulation (10 μg/ml, 10 minute incubation) did not affect the production of the D-3 phosphoinositide PtdIns(3)P consistently. [^{32}P] - PtdIns(3)P levels were slightly raised above vehicle treated controls in quiescent T lymphoblasts (**Fig. 3.20 a)**), a fold increase of 3.2 $\pm$ 1.3 was detected, but not in primary T lymphocytes (n = 2)(**Fig. 3.20 b)**). RANTES (1 ng/ml, 30 - 600 second incubation) did not increase [^{32}P] - PtdIns(3)P levels (n = 3).

[^{32}P] - PtdIns(3,4)P_2 levels were raised after stimulation with UCHT1 (10 μg/ml, 10 minute incubation) of quiescent T lymphoblasts (**Fig. 3.21 a)**) but not primary T lymphocytes (n = 3)(**Fig. 3.21 b)**). The increase in [^{32}P] - PtdIns(3,4)P_2 levels after UCHT1 stimulation of quiescent T lymphoblasts was 2.2 $\pm$ 0.75 fold (n = 4). However, RANTES (1 ng/ml, 30 - 600 second incubation) did not produce any consistent effects on [^{32}P] - PtdIns(3,4)P_2 levels (n = 2).

UCHT1 (10 μg/ml, 10 minute incubation) did cause an increase in [^{32}P] - PtdIns(3,4,5)P_3 levels in quiescent T lymphoblasts, with a 4.78 $\pm$ 1.9 fold increase above vehicle control levels (n = 2)(**Fig. 3.22 a)**), but no detectable effect on primary T lymphocyte [^{32}P] - PtdIns(3,4,5)P_3 levels (**Fig. 3.22 b)**). RANTES (1 ng/ml, 30 - 600 second incubation) produced no increase above vehicle control levels in quiescent T lymphoblasts and primary T lymphocytes.

3.2.3 Effect of RANTES on *In Vitro* PI 3-Kinase Activity

D-3 phosphoinositide production was not detected after RANTES stimulation of primary T lymphocytes, so an alternative approach was used to determine whether RANTES, at concentrations found to be optimal for chemotaxis, could increase PI 3-kinase activity associated with PI 3-kinase immunoprecipitates. An analysis of the dose-response effect of RANTES (0.01 - 1000 ng/ml) revealed that low concentrations of RANTES (0.01 - 1 ng/ml) produced a marked increase in PI 3-kinase activity in primary T lymphocytes, that was greater than that produced by the known PI 3-kinase activator, UCHT1 (10 μg/ml)(**Fig. 3.23**). Greater increases in PI 3-kinase activity were produced by 10 to 10000 ng/ml RANTES, with optimal responses following 10 to 100 ng/ml RANTES (n = 3).

In vitro kinase lipid activity of immunoprecipitates from RANTES (10 ng/ml) - stimulated primary T lymphocytes was increased in a time dependent manner (**Fig. 3.24**). Elevation of PI 3-kinase activity above basal levels was evident 30 seconds post-RANTES and a peak increase in activity was observed 600 seconds post-RANTES. PI

3-kinase activity had declined to 50% of peak value by 1800 seconds. This evidence implies that RANTES signalling is coupled to the PI 3-kinase signalling pathway.

3.2.4 Effects of Wortmannin on Primary T Lymphocyte Activation

To determine whether RANTES coupling to PI 3-kinase is functionally relevant a specific PI 3-kinase inhibitor, such as wortmannin, was used in the RANTES functional assays.

Wortmannin (0.01 - 1000 nM) inhibits RANTES-induced migration of primary T lymphocytes. The T lymphocytes were pretreated with wortmannin for 10 minutes before being stimulated with RANTES (1 ng/ml) in the microchemotaxis assay. The inhibition of RANTES-induced migration by wortmannin was concentration dependent and also dependent upon the concentration of RANTES used (**Fig. 3.25**). Hence, wortmannin had an IC_{50} of 0.04 $\pm$ 0.01 nM (n = 3) and 0.11 $\pm$ 0.08 nM (n = 3), against 1 ng/ml and 10 ng/ml RANTES, respectively.

The inhibition of RANTES induced migration of primary T lymphocytes was not due to the ethyl acetate vehicle in which wortmannin was reconstituted. T lymphocytes were incubated with vehicle or ethyl acetate at concentrations corresponding to the ethyl acetate concentration present in the relative wortmannin solution, before stimulation with 1 ng/ml RANTES in a microchemotaxis assay (**Fig. 3.26**). The ethyl acetate vehicle controls did not inhibit the RANTES induced migration.

Wortmannin inhibited the polarising effects of RANTES on primary T lymphocytes,

purified by plastic adherence (**Fig. 3.27**). Primary T lymphocytes were pretreated with wortmannin (0.1 - 100 nM) for 10 minutes, and incubated with RANTES (10 ng/ml) for 1 hour. Wortmannin inhibited the polarising effect of RANTES over the 0.1 - 100 nM concentration range, with an IC_{50} of 0.5 $\pm$ 0.12 nM (n = 3). Total inhibition of RANTES-mediated increases in polarisation was seen, with concentrations of wortmannin between 1 and 10 nM.

RANTES-induced actin polymerisation was also inhibited by wortmannin pretreatment (**Fig. 3.28**). Primary T lymphocytes were pretreated with wortmannin (0.001 - 1000 nM) for 10 minutes then incubated with 10 ng/ml RANTES for 10 minutes. The inhibitory effects of wortmannin on RANTES-induced actin polymerisation were dose dependent with an IC_{50} of 0.1 nM. Maximal inhibition was detected over the wortmannin concentration range of 1- 100 nM (n = 3).

The effect of wortmannin on RANTES-induced PI 3-kinase activity in primary T lymphocytes was examined by pretreating the cells with wortmannin (0.01 - 1000 nM), followed by stimulation with 10 ng/ml RANTES for 10 minutes (**Fig. 3.29**). The induction of PI 3-kinase activity induced by RANTES was inhibited by wortmannin in a concentration dependent manner. Complete inhibition was achieved, with a concentration between 10 and 100 nM (n = 3).

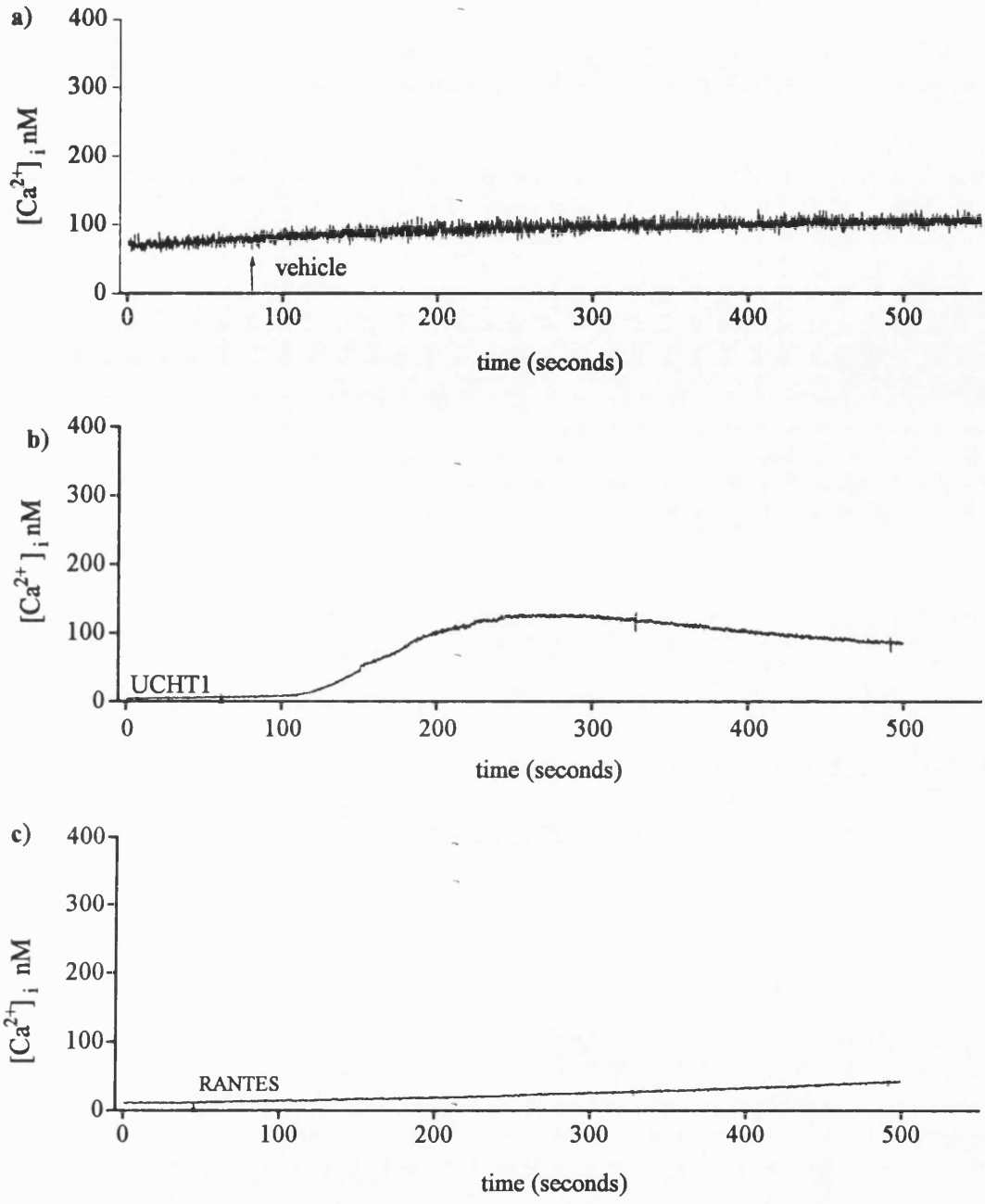


Fig. 3.14 Time Course of $[Ca^{++}]_i$ in Fura-2 Loaded Jurkat T Cells Stimulated with UCHT1 and RANTES

Time course of $[Ca^{2+}]_i$ in fura-2 loaded Jurkat T cells, as determined by the Photon Technology International software program, for formulae see Section 2.9. The cells were activated with **a)** vehicle control, **b)** UCHT1 (10 μg/ml), **c)** 10 ng/ml RANTES. The traces were obtained from one experiment, but are representative of three other experiments.

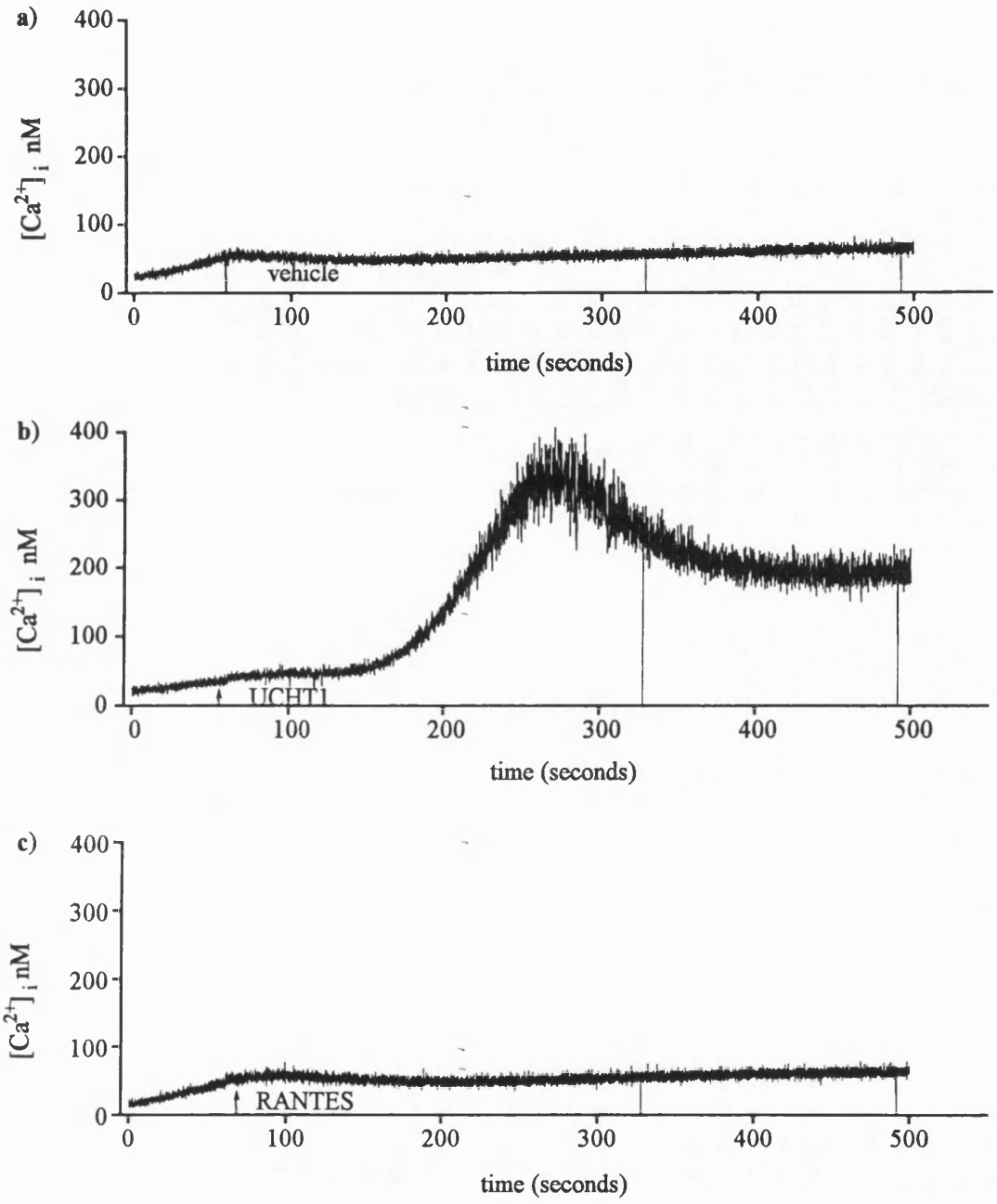


Fig. 3.15 Time Course of $[Ca^{++}]_i$ in fura-2 Loaded Quiescent T Lymphoblasts Stimulated with UCHT1 and RANTES

Time course of $[Ca^{2+}]_i$ in fura-2 loaded quiescent T lymphoblasts, as determined by the Photon Technology International software program. The cells were activated with **a)** vehicle control, **b)** UCHT1 (10 μg/ml), **c)** 10 ng/ml RANTES. The traces were obtained from one experiment, but are representative of three other experiments.

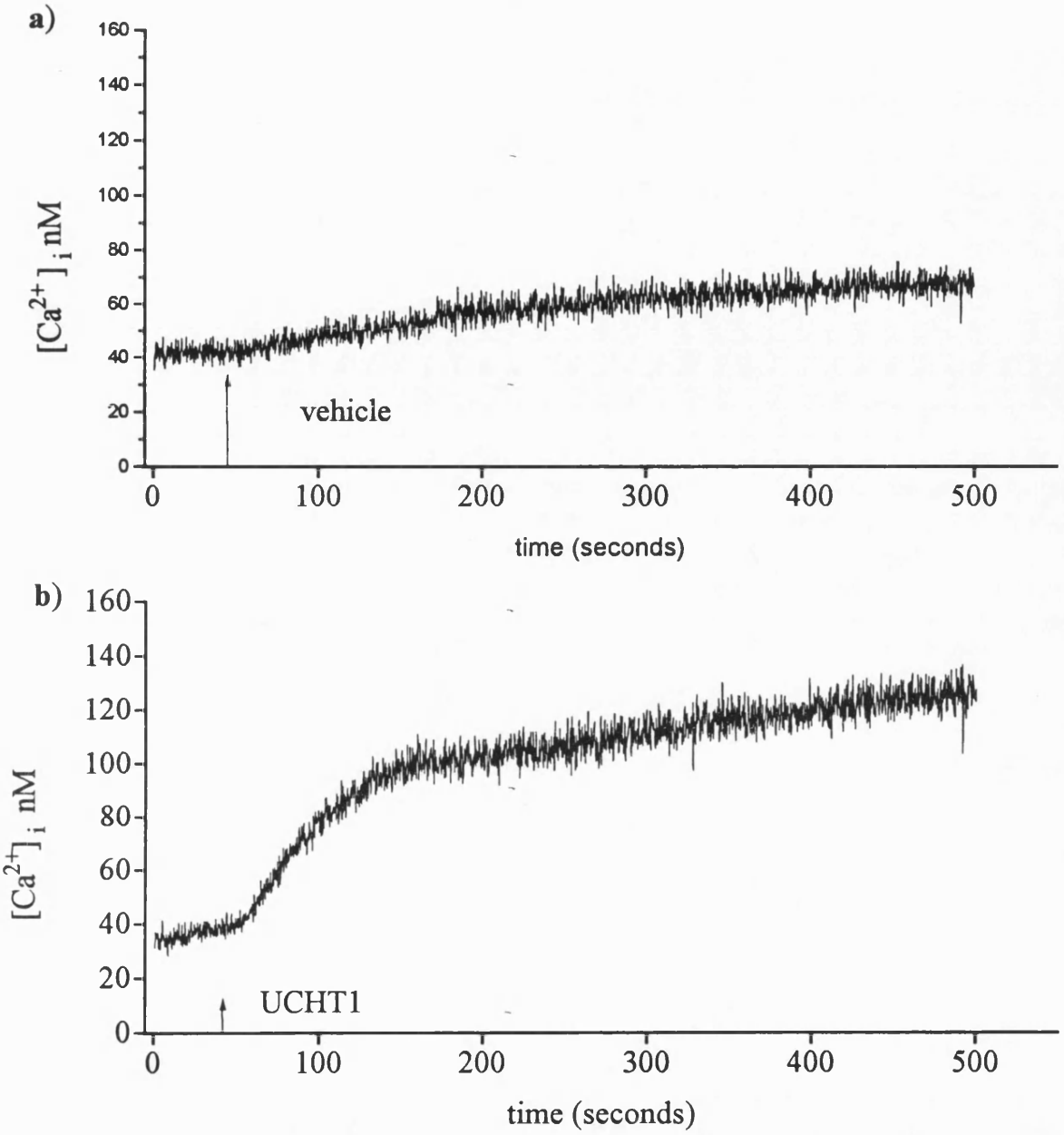


Fig. 3.16 Time Course of $[Ca^{++}]_i$ in fura-2 Loaded Primary T Lymphocytes Stimulated with UCHT1

Time course of $[Ca^{2+}]_i$ in fura-2 loaded primary T lymphocyte (purified by plastic adherence), as determined by the Photon Technology International software program. The cells were activated with **a)** vehicle control, and **b)** UCHT1 (10 μg/ml). The traces were obtained from one experiment, but are representative of three other experiments.

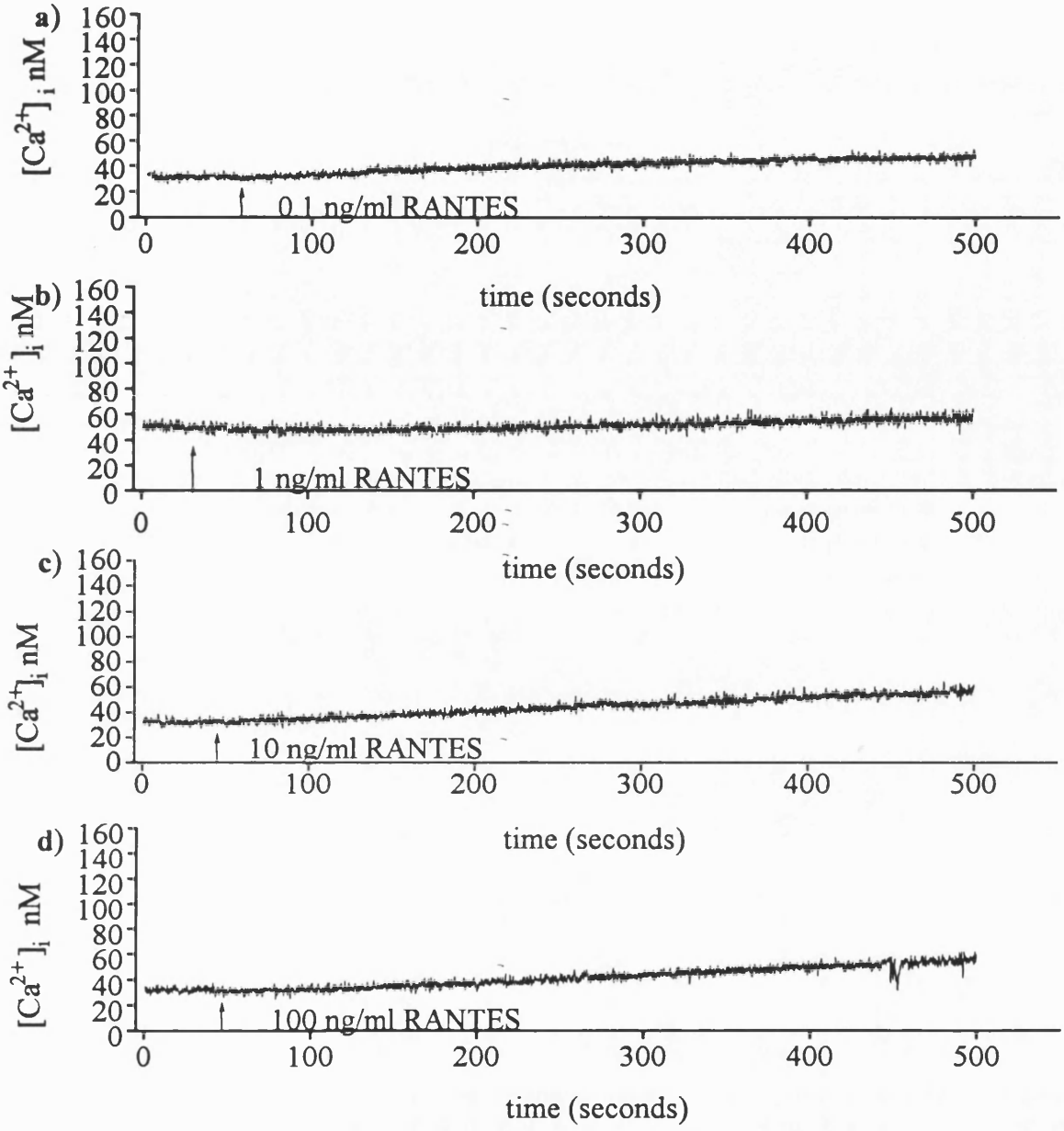


Fig. 3.17 Time Course of $[Ca^{++}]_i$ in fura-2 Loaded Primary T Lymphocytes Stimulated with RANTES

Time course of $[Ca^{2+}]_i$ in fura-2 loaded primary T lymphocytes (purified by plastic adherence) as determined by the Photon Technology International software program. The cells were activated with **a)** 0.1 ng/ml RANTES, **b)** 1 ng/ml RANTES, **c)** 10 ng/ml RANTES, and **d)** 100 ng/ml RANTES. The control experimental results are shown in **Fig. 3.15**. The traces were obtained from one experiment, but are representative of three other experiments.

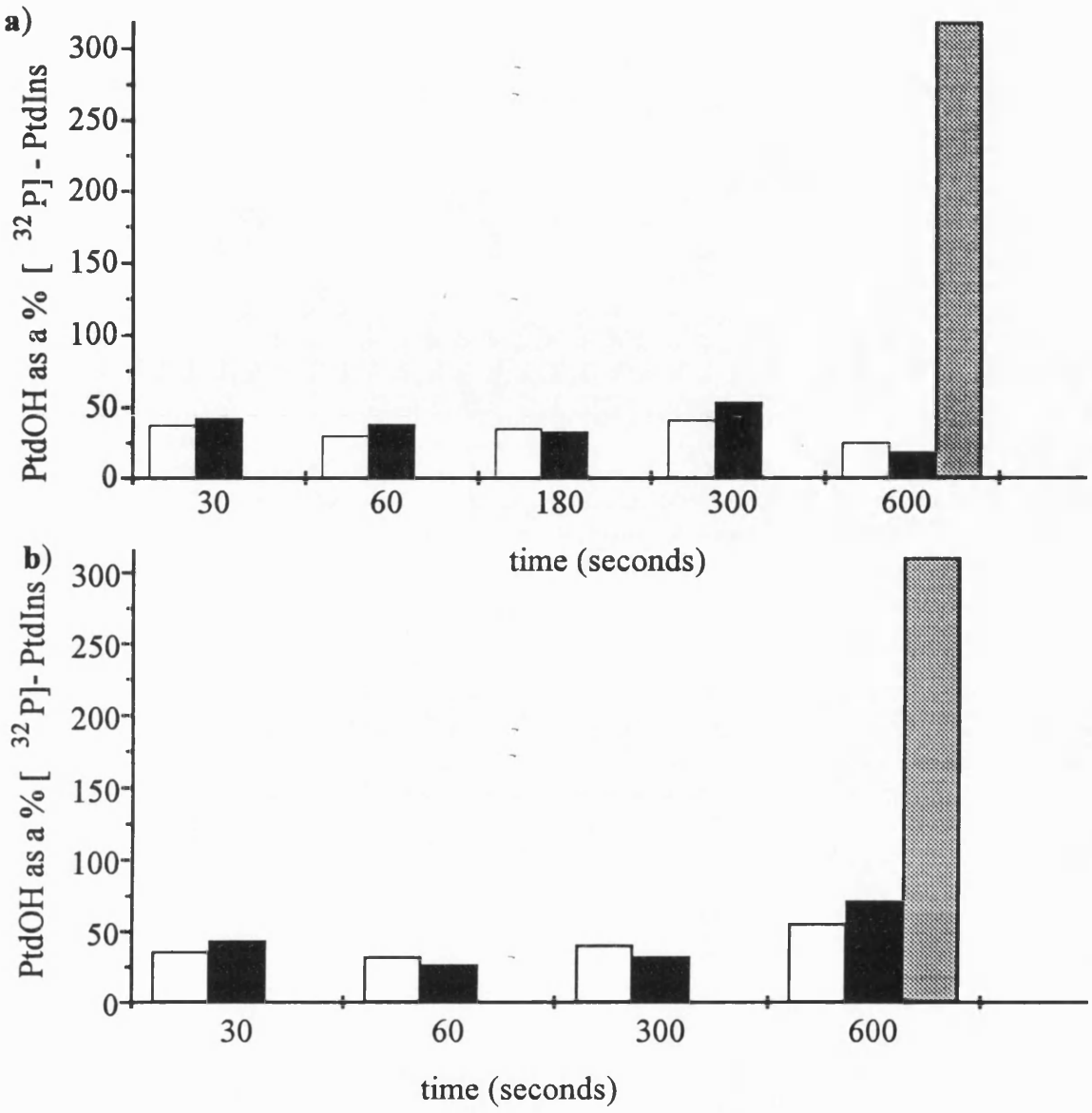


Fig. 3.18 Effect of UCHT1 and RANTES on [^{32}P] - PtdOH Production in Quiescent T Lymphoblasts and Primary T Lymphocytes

Effect of vehicle control (30 - 600 second incubation, □), UCHT1 (10 μg/ml, 10 minute incubation, ▩) and RANTES (1 ng/ml, 30 - 600 second incubation, ■) on [^{32}P] - PtdOH production in **a)** quiescent T lymphoblasts and **b)** primary T lymphocytes, purified by plastic adherence. Phosphatidylinositol metabolism was analysed as described in Materials and Methods. All results are expressed as a percentage of [^{32}P] - PtdIns detected in the same sample. Results are from a single representative experiment of at least two others performed.

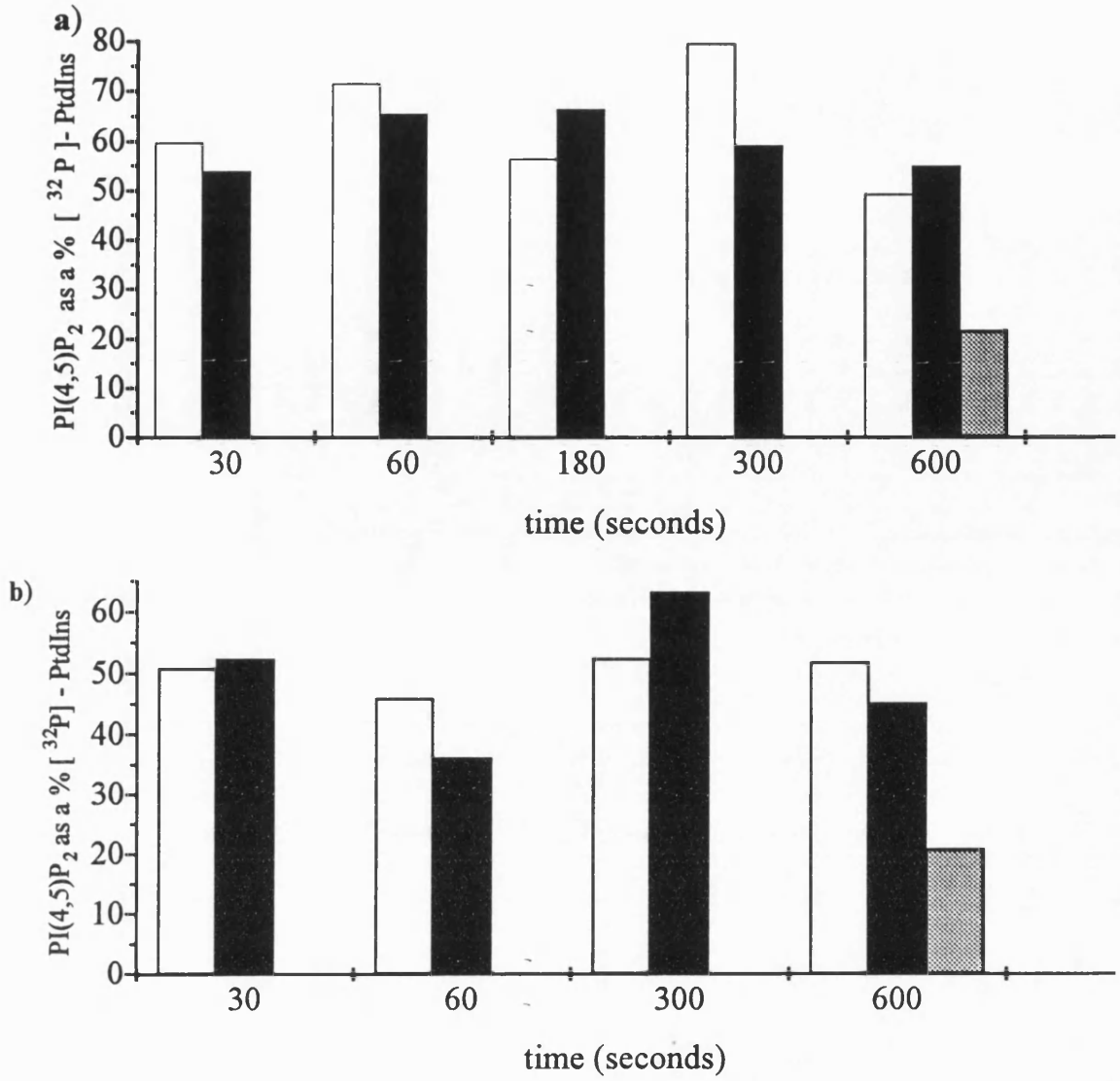


Fig. 3.19 Effect of UCHT1 and RANTES on [^{32}P] - PtdIns(4,5)P_2 Breakdown in Quiescent T Lymphoblasts and Primary T Lymphocytes

Effect of vehicle control (30 - 600 second incubation, □), UCHT1 (10 μg/ml, 10 minute incubation, ▩) and RANTES (1 ng/ml, 30 - 600 second incubation, ■) on [^{32}P] - PtdIns(4,5)P_2 breakdown in **a)** quiescent T lymphoblasts and **b)** primary T lymphocytes, purified by plastic adherence. Phosphatidylinositol metabolism was analysed as described in Materials and Methods. All results are expressed as a percentage of [^{32}P] - PtdIns detected in the same sample. Results are from a single representative experiment of at least two others performed.

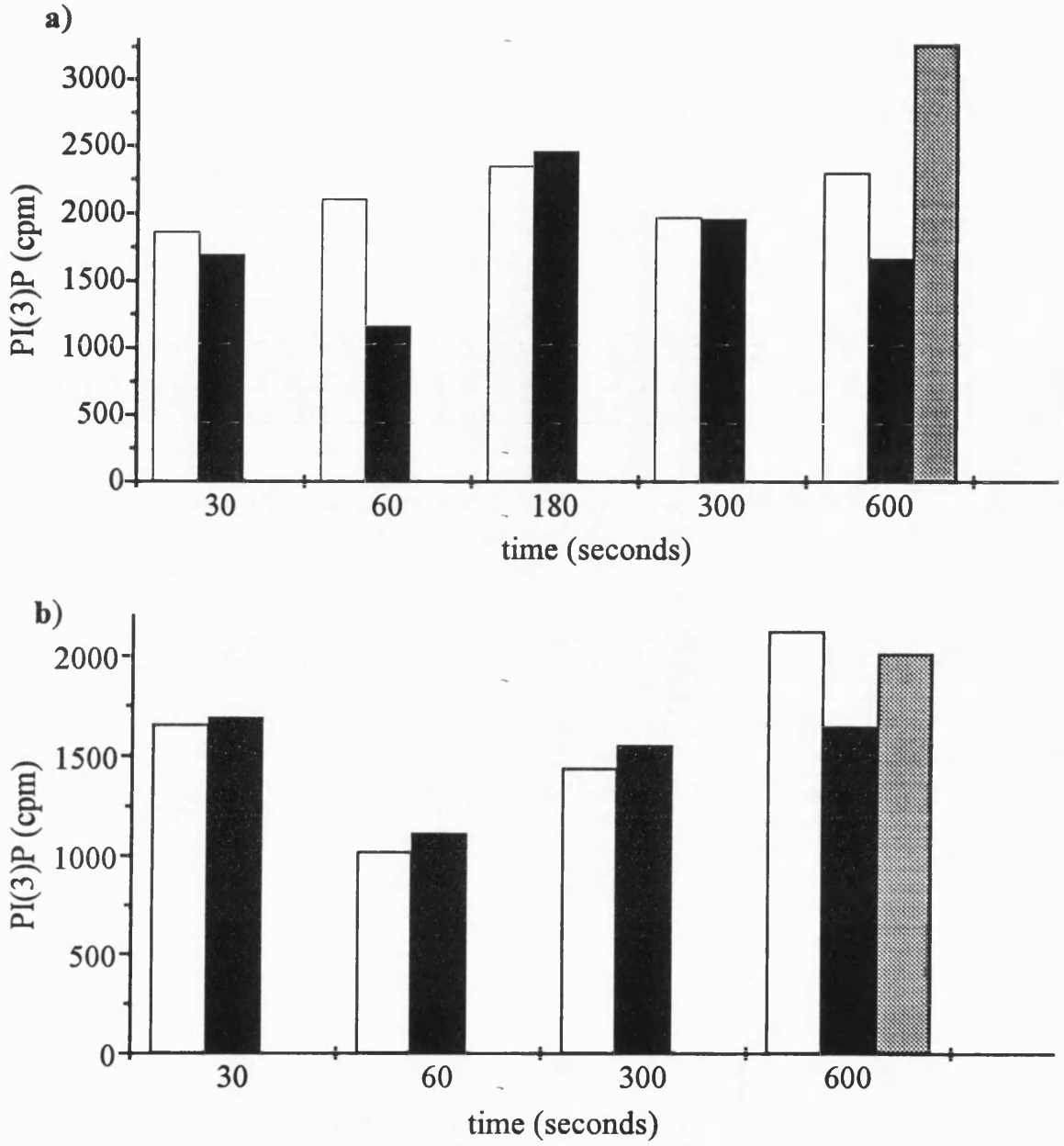


Fig. 3.20 Effect of UCHT1 and RANTES on [^{32}P] - PtdIns(3)P Production in Quiescent T Lymphoblasts and Primary T Lymphocytes

Effect of vehicle control (30 - 600 second incubation, □), UCHT1 (10 μg/ml, 10 minute incubation, ▩) and RANTES (1 ng/ml, 30 - 600 second incubation, ■) on [^{32}P] - PtdIns(3)P production in **a)** quiescent T lymphoblasts and **b)** primary T lymphocytes, purified by plastic adherence. Phosphatidylinositol metabolism was analysed as described in Materials and Methods. All results are expressed in cpm. Results are from a single representative experiment of at least two others performed.

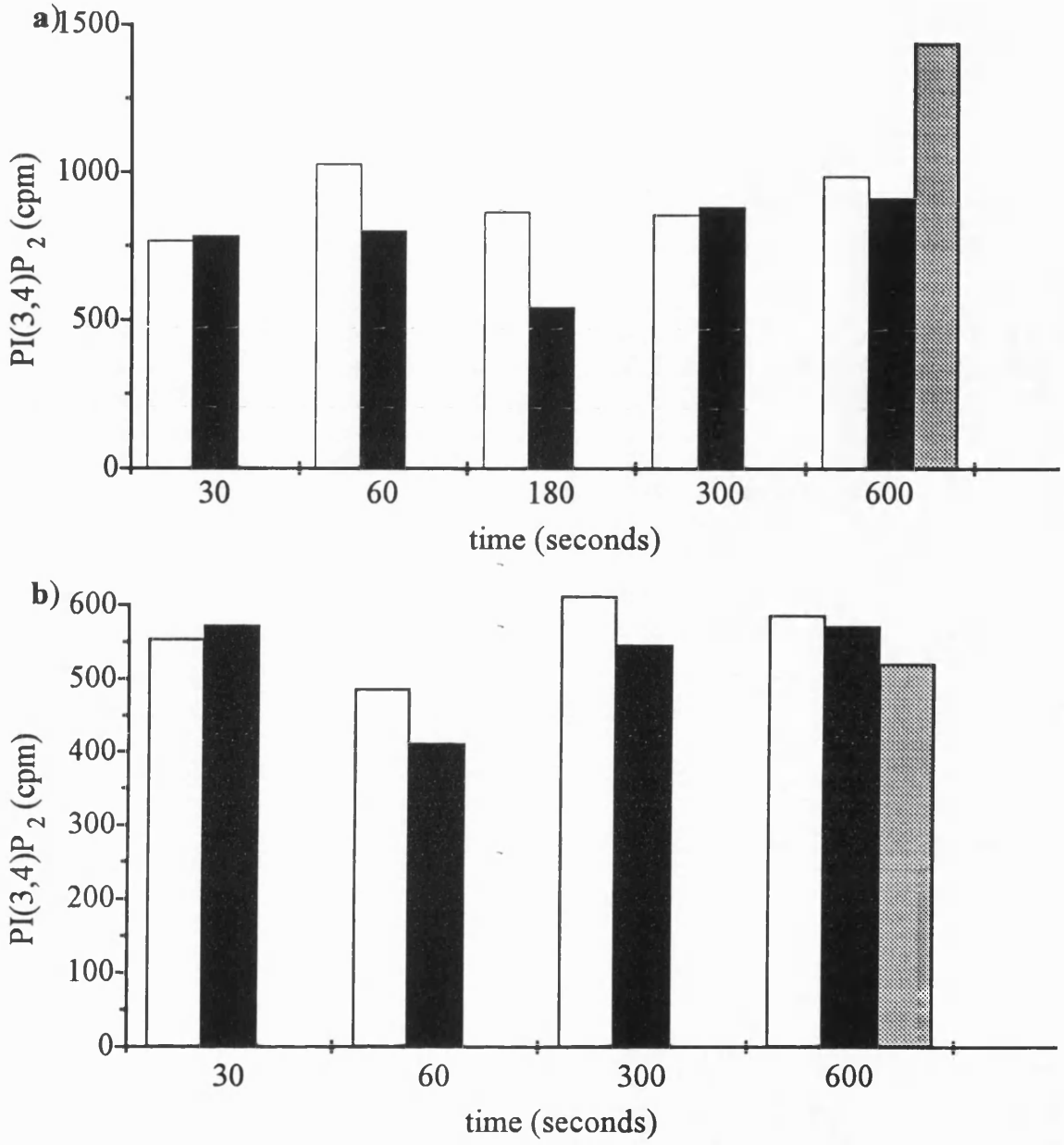


Fig. 3.21 Effect of UCHT1 and RANTES on [^{32}P] - PtdIns(3,4)P$_2$ Production in Quiescent T Lymphoblasts and Primary T Lymphocytes

Effect of vehicle control (30 - 600 second incubation, □), UCHT1 (10 μg/ml, 10 minute incubation, ▩) and RANTES (1 ng/ml, 30 - 600 second incubation, ■) on [^{32}P] - PtdIns(3,4)P$_2$ production in a) quiescent T lymphoblasts and b) primary T lymphocytes, purified by plastic adherence. Phosphatidylinositol metabolism was analysed as described in Materials and Methods. All results are expressed in cpm. Results are from a single representative experiment of at least two others performed.

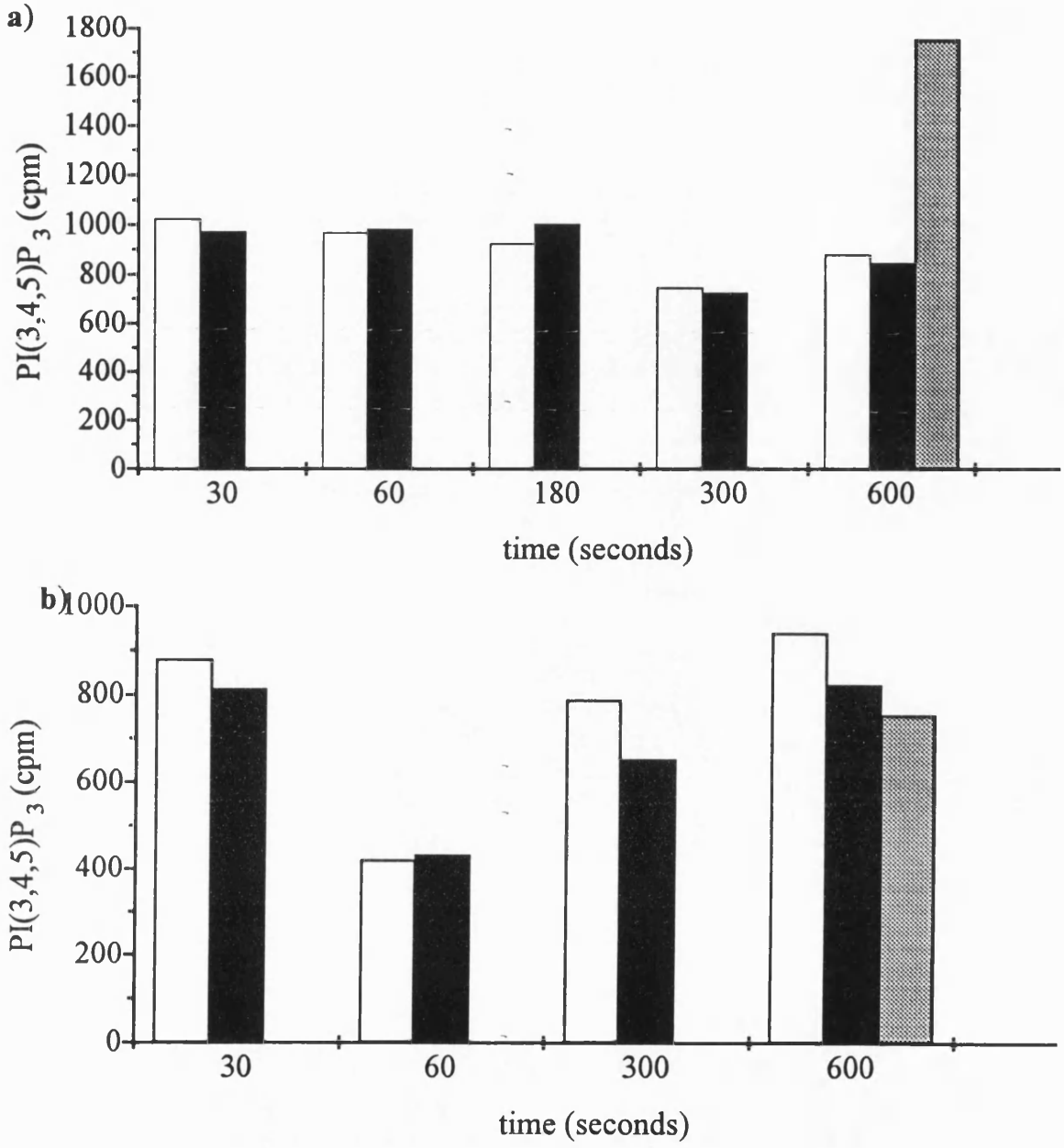


Fig. 3.22 Effect of UCHT1 and RANTES on [^{32}P] - PtdIns(3,4,5)P_3 Production in Quiescent T Lymphoblasts and Primary T Lymphocytes

Effect of vehicle control (30 - 600 second incubation, □), UCHT1 (10 μg/ml, 10 minute incubation, ▩) and RANTES (1 ng/ml, 30 - 600 second incubation, ■) on [^{32}P] - PtdIns(3,4,5)P_3 production in a) quiescent T lymphoblasts and b) primary T lymphocytes, purified by plastic adherence. Phosphatidylinositol metabolism was analysed as described in Materials and Methods. All results are expressed in cpm. Results are from a single representative experiment of at least two others performed.

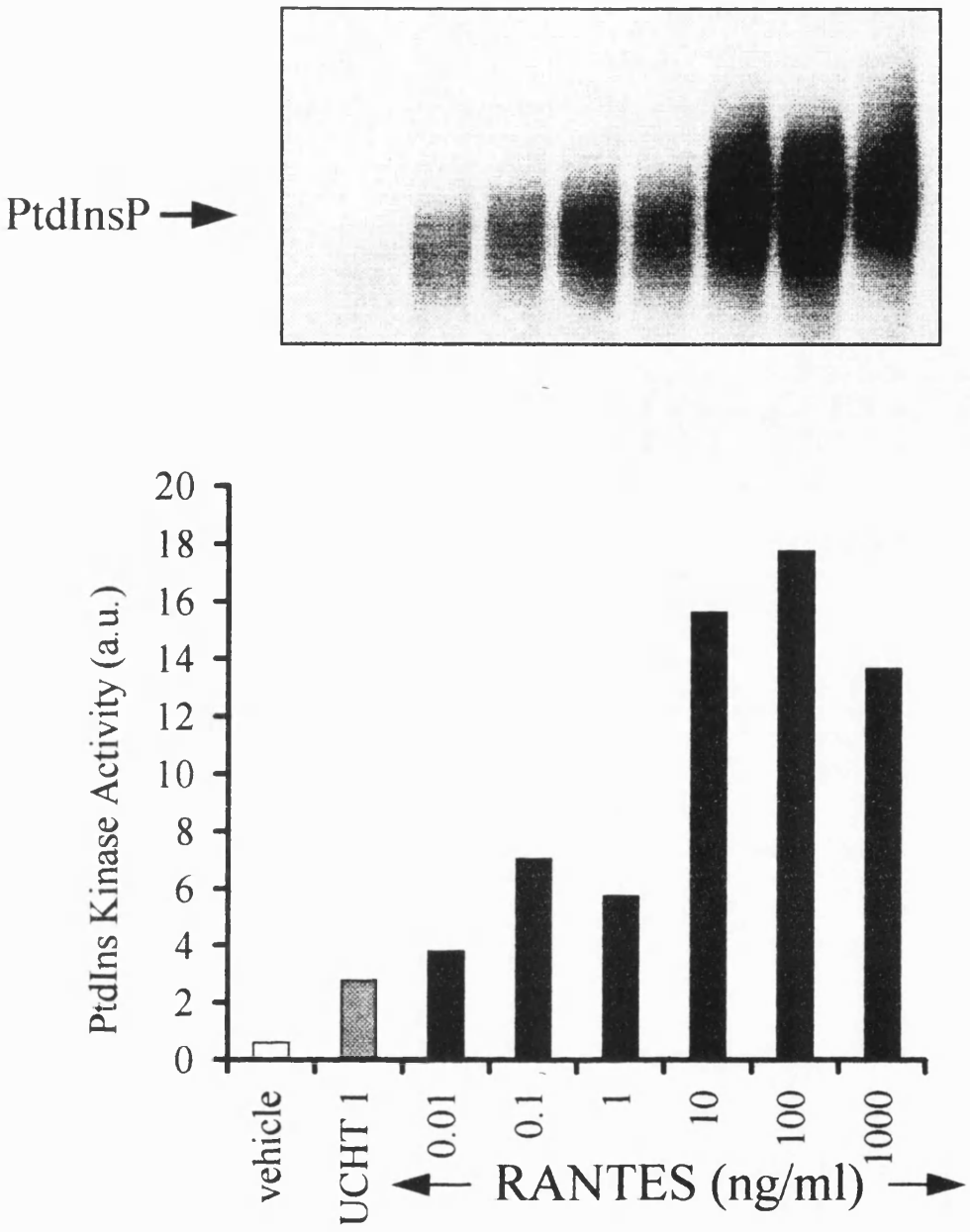


Fig. 3.23 Effect of RANTES on PI 3-Kinase Activity - dose response curve

Effect of RANTES on PI 3-kinase activity in T lymphocytes, purified by plastic adherence. T lymphocytes were stimulated by RANTES (0.01 - 1000 ng/ml, ■), UCHT1 (10 μg/ml, ▩), or vehicle control (□) for 10 minutes. The PI 3-kinase from cell lysates was immunoprecipitated, an *in vitro* lipid kinase assay was conducted and the labelled lipids were analysed, see Materials and Methods. The upper panel is a TLC autoradiograph and the lower panel is a densitometric scan of the autoradiograph in arbitrary units (a.u.).

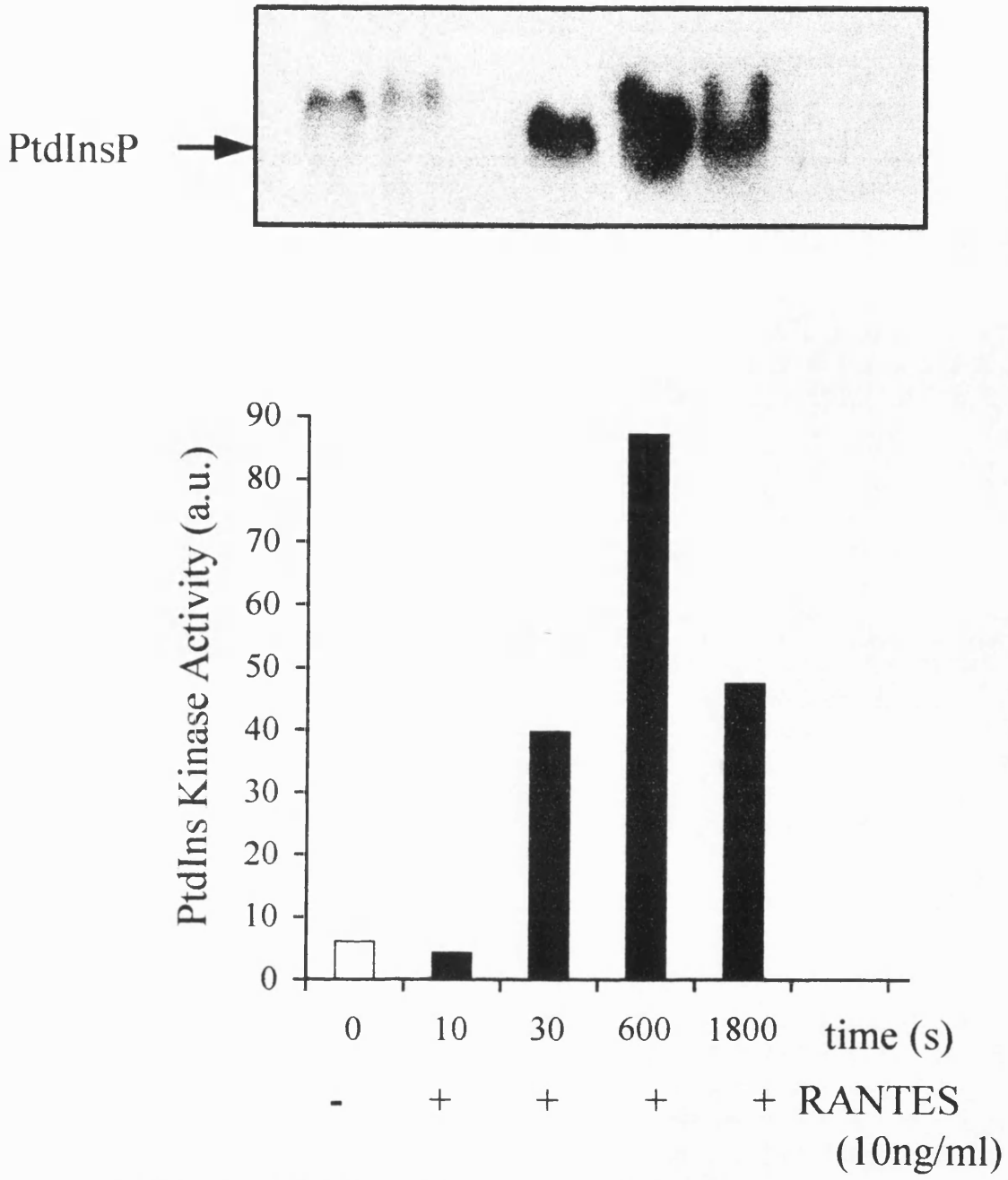


Fig. 3.24 Effect of RANTES on PI 3-Kinase Activity - time course

Effect of RANTES over a time course of 10 to 1800 seconds on PI 3-kinase activity in T lymphocytes, purified by plastic adherence. T lymphocytes were stimulated by 10 ng/ml RANTES (■), or vehicle control (□) for 10 minutes. The PI 3-kinase from cell lysates was immunoprecipitated, an *in vitro* lipid kinase assay was conducted and the labelled lipids were analysed, see Materials and Methods. The upper panel is a TLC autoradiograph and the lower panel is a densitometric scan of the autoradiograph in arbitrary units (a.u.).

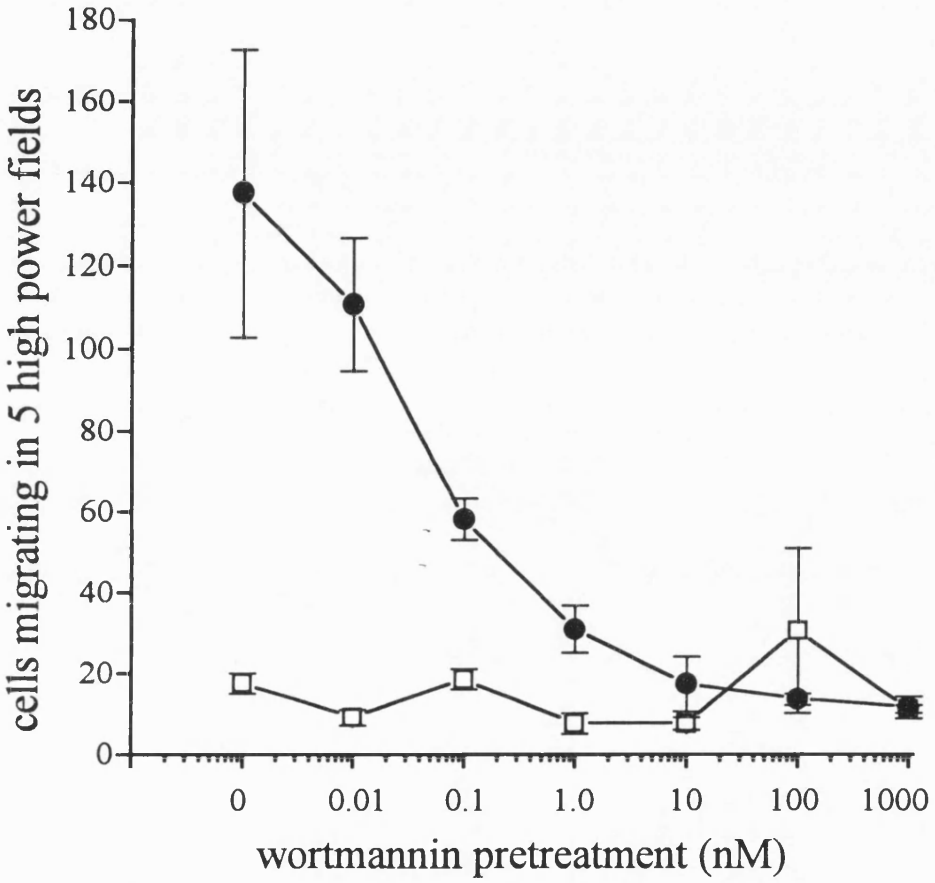


Fig. 3.25 Effect of Wortmannin on RANTES-Induced Migration of T Lymphocytes

Effect of wortmannin on RANTES-induced migration of T lymphocytes. T lymphocytes, purified by plastic adherence, were pretreated with wortmannin (0.01 - 1000 nM) for 10 minutes. The cells were then incubated with 1 ng/ml RANTES (●) or vehicle control (□) in a 48-well microchemotaxis chamber for 4 hours, as described in Materials and Methods. Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least three others performed.

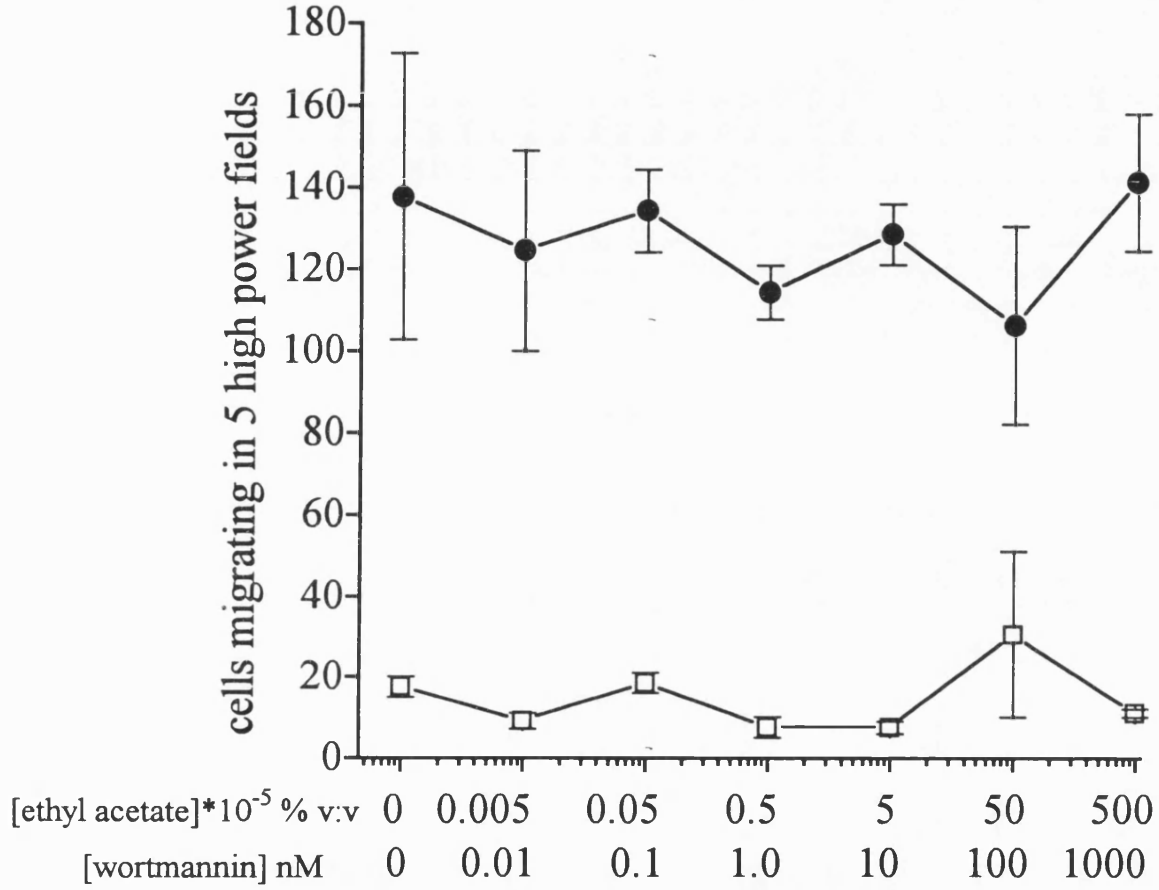


Fig. 3.26 Effect of Ethyl Acetate Vehicle on RANTES-Induced Migration of T Lymphocytes

Effect of ethyl acetate vehicle on RANTES-induced migration of T lymphocytes. T lymphocytes, purified by plastic adherence, were pretreated with % ethyl acetate at concentrations corresponding to the concentrations present in corresponding wortmannin solutions (0.01 - 1000 nM) for 10 minutes. The cells were then incubated with 1 ng/ml RANTES (●) or vehicle control (□) in a 48-well microchemotaxis chamber for 4 hours, as described in Materials and Methods. Results are expressed as the mean number (± SEM) of migrating cells in 5 high power magnification fields (400 x), from triplicate wells. Results are from a single representative experiment of at least one other performed.

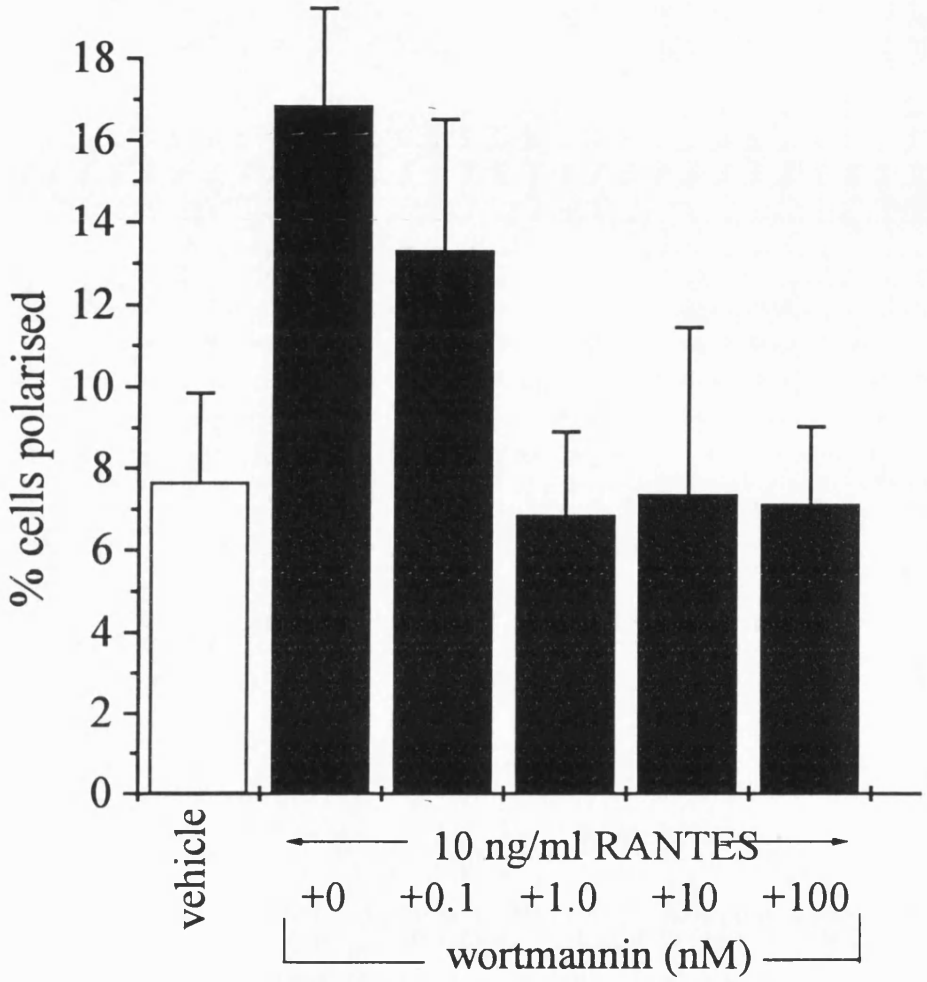


Fig. 3.27 Effect of Wortmannin on RANTES-Induced Polarisation of T Lymphocytes

Effect of wortmannin on RANTES-induced polarisation of T lymphocytes. T lymphocytes, purified by plastic adherence, were pretreated with wortmannin (0.1 - 100 nM) for 10 minutes. The cells were then incubated with 10 ng/ml RANTES (■) or vehicle control (□) for 60 minutes. Results are expressed as the proportion of T lymphocytes that polarise after culture with RANTES. Each treatment was repeated in triplicate and each point is the mean (± SEM) of these triplicates. Results are from a single representative experiment of three others performed.

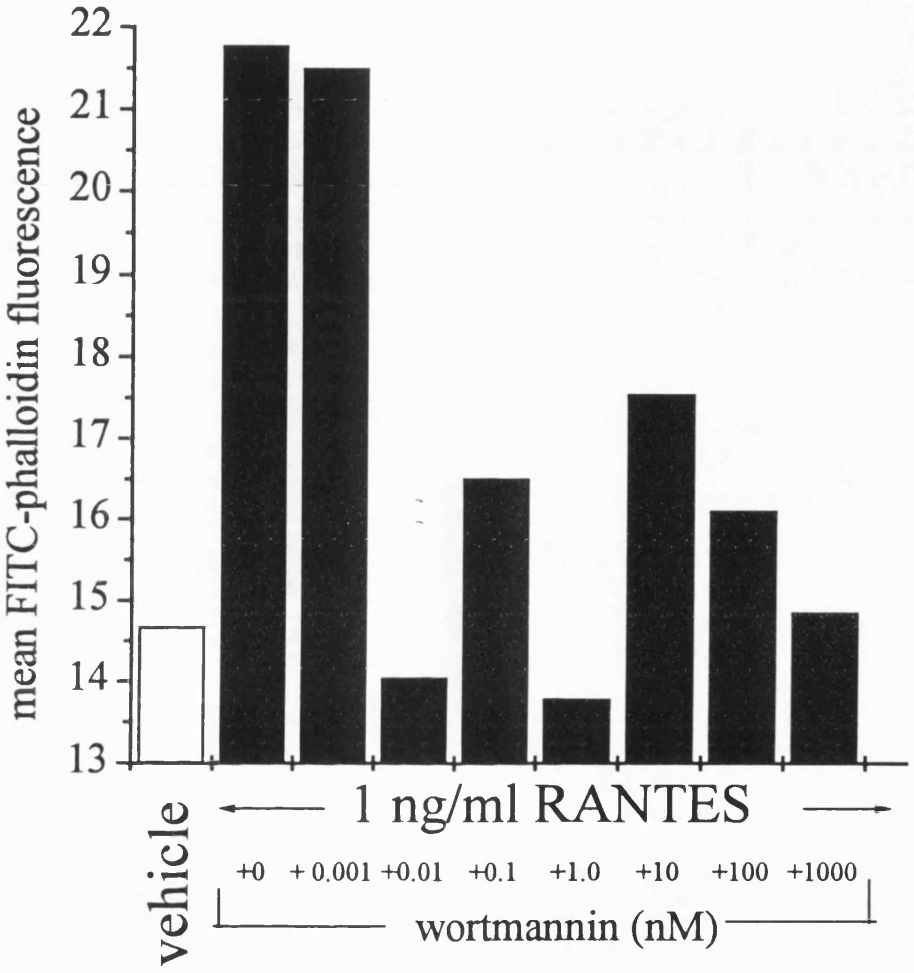


Fig. 3.28 Effect of Wortmannin on RANTES-Induced Actin Polymerisation of T Lymphocytes

Effect of wortmannin on RANTES-induced actin polymerisation of T lymphocytes. T lymphocytes, purified by plastic adherence, were pretreated with wortmannin (0.001 - 1000 nM) for 10 minutes. The cells were then incubated with 10 ng/ml RANTES (■) or vehicle control (□), fixed, and the degree of actin polymerisation was assessed using FITC-phalloidin, which only binds to F-actin, not the unpolymerised form, see Materials and Methods. Results are from a single representative experiment of three others performed.

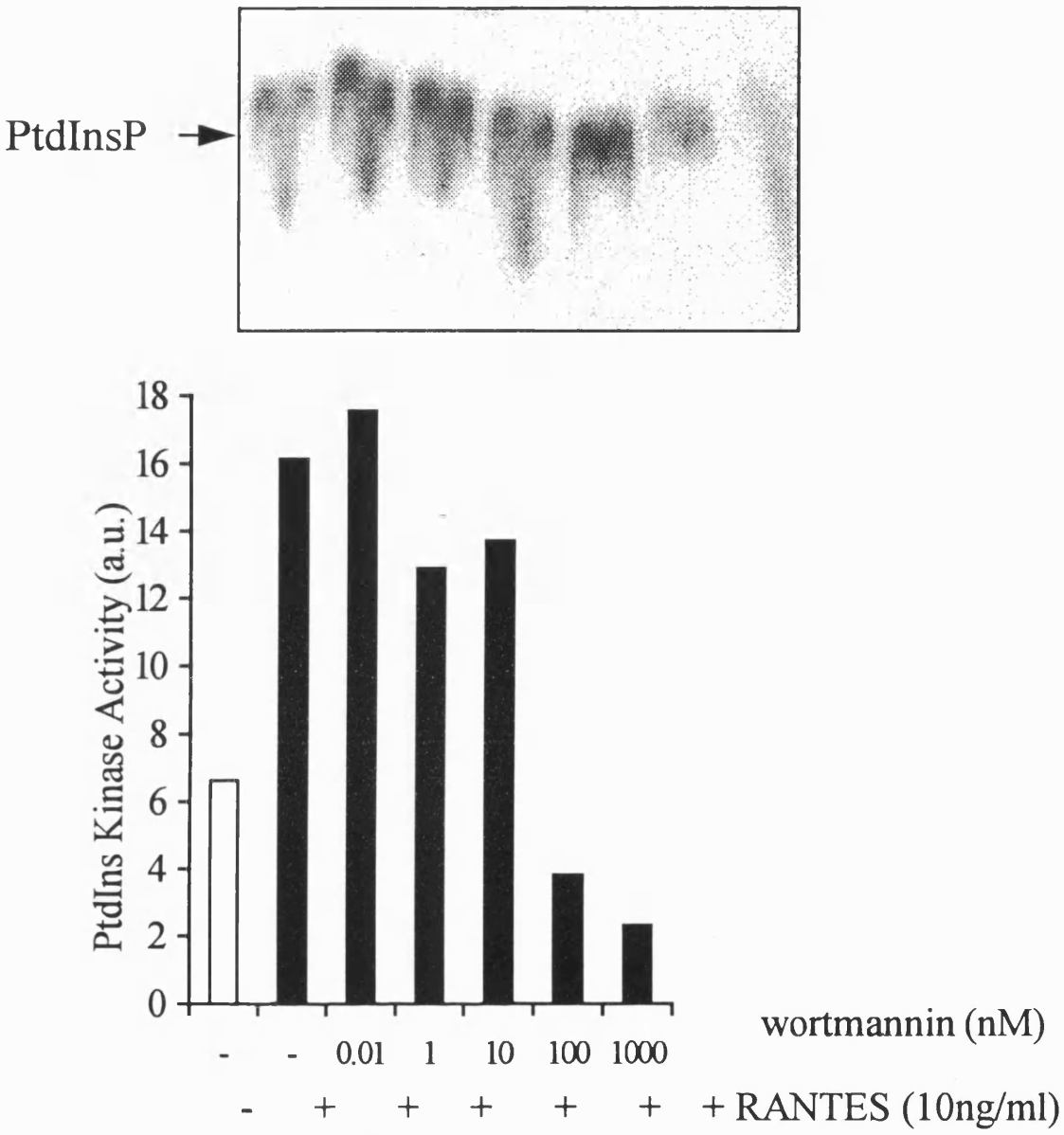


Fig. 3.29 Effect of Wortmannin on RANTES-Induced PI 3-Kinase Activity

Effect of wortmannin on RANTES-induced PI 3-kinase activity of T lymphocytes. T lymphocytes, purified by plastic adherence, were pretreated with wortmannin (0.01 - 1000 nM) for 10 minutes. The cells were then stimulated by 10 ng/ml RANTES (■), or vehicle control (□) for 10 minutes. The PI 3-kinase from cell lysates was immunoprecipitated, an *in vitro* lipid kinase assay was conducted and the labelled lipids were analysed, see Materials and Methods. The upper panel is a TLC autoradiograph and the lower panel is a densitometric scan of the autoradiograph in arbitrary units (a.u.).

3.3 Costimulation and RANTES

3.3.1 Modulation of T Lymphocyte Proliferation by RANTES

Treatment of T lymphocytes, purified with a R+D column (which enables quicker preparation of the cells), **a)** with UCHT1 alone (0.01 - 10 μg/ml) or **b)** treatment with fixed CHO-B7$^+$ cells alone (1 : 7 T cells - 1 : 1 T cells) did not increase T lymphocyte proliferation consistently , although 1 - 10 μg/ml UCHT1 did increase proliferation by a factor of 2 above vehicle control levels **(Fig. 3.30)**.

Costimulation of the T lymphocytes with 0.01 μg/ml UCHT1 and fixed CHO-B7$^+$ cells (CHO-B7$^+$: T cell of 1:7, 1:5, 1:3, 1:1) did not induce proliferation but modulation of proliferation was observed with 0.1 μg/ml UCHT1 plus CHO-B7$^+$. 1 μg/ml UCHT1 was more effective at inducing proliferation in combination with CHO-B7$^+$ cells but the most effective stimulus was 10 μg/ml UCHT1 plus CHO-B7$^+$ cells. A ratio of 1 : 1 CHO-B7$^+$ cells : T cells in combination with 10 μg/ml UCHT1 caused the greatest increase in proliferation of T lymphocytes, but this ratio was difficult to use as large numbers of CHO-B7$^+$cells were required **(Fig. 3.31)**. Therefore a combination of 10 μg/ml UCHT1 plus 1 CHO-B7$^+$: 3 T cells was used as the optimal costimulus.

Again, there were differences in the extent of proliferation seen with different donors, but the trends were always comparable between the different populations, e.g. 10 μg/ml anti-CD3 and 1 CHO-B7$^+$: 3 T cells induced a 8.3 $\pm$ 1.7 fold increase in proliferation (n = 4).

RANTES (0.01 - 100 ng/ml) did not increase proliferation of T lymphocytes above levels detected with vehicle treatment only (**Fig. 3.32 a)**). In combination with 10 μg/ml UCHT1, RANTES did not raise the level of proliferation seen with UCHT1 and vehicle only (**Fig. 3.32 b)**). Also, in combination with CHO-B7$^+$ cells, RANTES did not increase proliferation above the level that was detected with CHO-B7$^+$ cells alone (**Fig. 3.32 c)**). RANTES (0.01 - 100 ng/ml) did, however, potentiate T lymphocyte proliferation when purified T lymphocytes were costimulated with UCHT1 plus CHO-B7$^+$ cells (**Fig. 3.33**). Peak potentiating effects were seen at 1 - 10 ng/ml RANTES. RANTES (1 ng/ml) produced a 55 $\pm$ 22.9 % (n = 4) potentiation of proliferation when added in combination with UCHT1 plus CHO-B7$^+$ cells.

T lymphocytes were costimulated with UCHT1 (10 μg/ml) plus CHO-B7$^+$ in the presence of monoclonal anti-RANTES (5 - 0.005 μg/ml) or a isotype matched control antibody (5 - 0.005 μg/ml). Treatment of the primary T lymphocytes with anti-RANTES inhibits proliferation (**Fig. 3.34**). Anti-RANTES (5 μg/ml) produced a 25 $\pm$ 5 % inhibition of proliferation (n = 4). The addition of an isotype matched control antibody was without effect. The inhibitory effects of anti-RANTES produced a curve that is the inverse of the characteristic "bell-shape-like" dose response curve which is normally seen with RANTES stimulation of T lymphocytes.

3.3.2 Costimulation of T Lymphocytes Modulates RANTES Peptide Production

Primary T lymphocytes were stimulated with either vehicle, UCHT1 (10 μg/ml), fixed CHO-B7$^+$ cells, or a combined costimulation of UCHT1 plus CHO-B7$^+$ cells for 0 - 48 hours (**Fig. 3.35**). The concentration of RANTES peptide produced was low in

vehicle treated T lymphocytes and cells treated with UCHT1 or CHO-B7$^+$ cells alone. The most effective stimulus for RANTES generation was a combination of UCHT1 plus CHO-B7$^+$ cells. The increase in RANTES production was seen at 24 hours and continued to rise throughout the time course for these experiments. A set of RANTES generation experiments were performed over a longer time course. T lymphocytes were stimulated with either vehicle or costimulation of UCHT1 plus CHO-B7$^+$ cells for 48 - 96 hours (**Fig. 3.36**). Cells treated with costimulation produced high levels of RANTES which plateaued out at 48 hours of treatment. The actual increase in RANTES peptide production was largely dependent upon the cell preparations used, with T lymphocyte populations from different donors having different degrees of peptide production but costimulation consistently induced the highest release of peptide.

3.3.3 Modulation of IL-2 Receptor by RANTES

Purified T lymphocytes were stimulated with vehicle, UCHT1 (10 μg/ml), CHO-B7$^+$, or UCHT1 plus CHO-B7$^+$ (**Fig. 3.37**). CD25 (IL-2 R) levels were increased after stimulation with UCHT1 plus CHO-B7$^+$. UCHT1 or CHO-B7$^+$ stimulation alone appeared to induce small increases in CD25 expression. RANTES (1 ng/ml) was then applied in addition to the previous treatments. RANTES in combination with the vehicle or UCHT1 did not potentiate CD25 expression. When T lymphocytes were stimulated with CHO-B7$^+$ and RANTES, CD25 expression was increased above levels seen with CHO-B7$^+$ alone. RANTES also potentiated the effect seen with UCHT1 plus CHO-B7$^+$ on CD25 expression.

RANTES, in combination with CHO-B7$^+$ or UCHT1 plus CHO-B7$^+$, switched on the expression of IL-2 R in cells that had been negative for this receptor, increasing the % total cells binding to FITC-labelled anti-CD25 (**Fig. 3.38**). RANTES potentiated the response seen to CH0-B7$^+$ stimulation, with a 2.4 $\pm$ 0.8 fold increase in FITC-labelled anti-CD25 binding cells (n = 4), and the response seen to costimulation with UCHT1 plus CHO-B7$^+$, with a 1.8 $\pm$ 0.6 fold increase (n = 4). In addition, RANTES, in combination with CHO-B7$^+$ or UCHT1 plus CHO-B7$^+$, upregulated the intensity of fluorescence of FITC-labelled anti-CD25 binding in cells constitutively expressing low levels of the marker, increasing the mean fluorescence per cell (**Fig. 3.39**).

3.3.4 Modulation of IL-2 Peptide Production by RANTES

Primary T lymphocytes were stimulated with either vehicle, UCHT1 (10 μg/ml), fixed CHO-B7$^+$ cells, or a combined costimulation of UCHT1 plus CHO-B7$^+$ cells for 48 hours (**Fig. 3.40**). The concentration of IL-2 produced was calculated using a IL-2 dependent cell line.

IL-2 production was low in vehicle treated T lymphocytes and cells treated with CHO-B7$^+$ alone. UCHT1 stimulation of the T lymphocytes did increase IL-2 production but this increase was variable between different donors. The most effective stimulus for IL-2 generation was a combination of UCHT1 plus CHO-B7$^+$ cells, with a 4.1 $\pm$ 1.9 fold rise observed above vehicle control levels (n = 3).

RANTES (1 ng/ml) was then applied in addition to the previous treatments. RANTES in combination with the vehicle or UCHT1 or CHO-B7$^+$ alone did not affect IL-2

production consistently, with small increases only observed in two out of four experiments and RANTES did not significantly potentiate the effect seen with UCHT1 plus CHO-B7$^+$ on IL-2 production. So RANTES had no effect on IL-2 production even in combination with CHO-B7$^+$ plus UCHT1, although it does potentiate IL-2 R upregulation, in this situation.

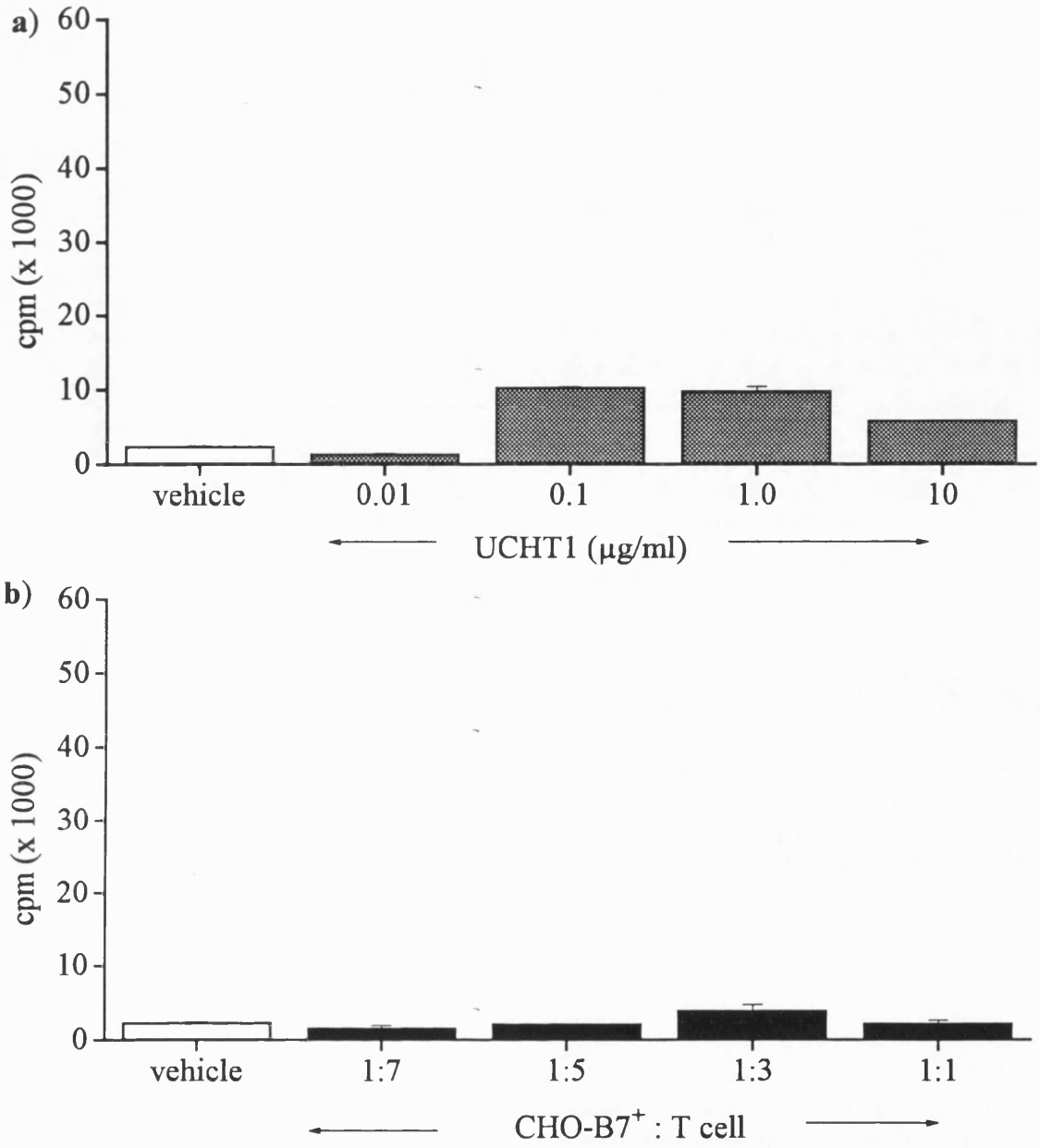


Fig. 3.30 Effect of UCHT1 and CHO-B7$^+$ on Proliferation of Primary T Lymphocytes

Effect of **a)** UCHT1 alone (0.01 - 10 μg/ml, ▩) or **b)** treatment with CHO-B7$^+$ as APC (CHO-B7$^+$: T cell of 1:7, 1:5, 1:3, 1:1, ■), or vehicle control (□) on proliferation of purified T lymphocytes. T lymphocytes were purified using a R+D column then treated with UCHT1 or CHO-B7$^+$ for 72 hours. Proliferation was measured by [^{3}H] - thymidine incorporation (cpm x 1000), see Materials and Methods production. Each treatment was repeated in quadruplicate and the mean (± SEM) is shown.

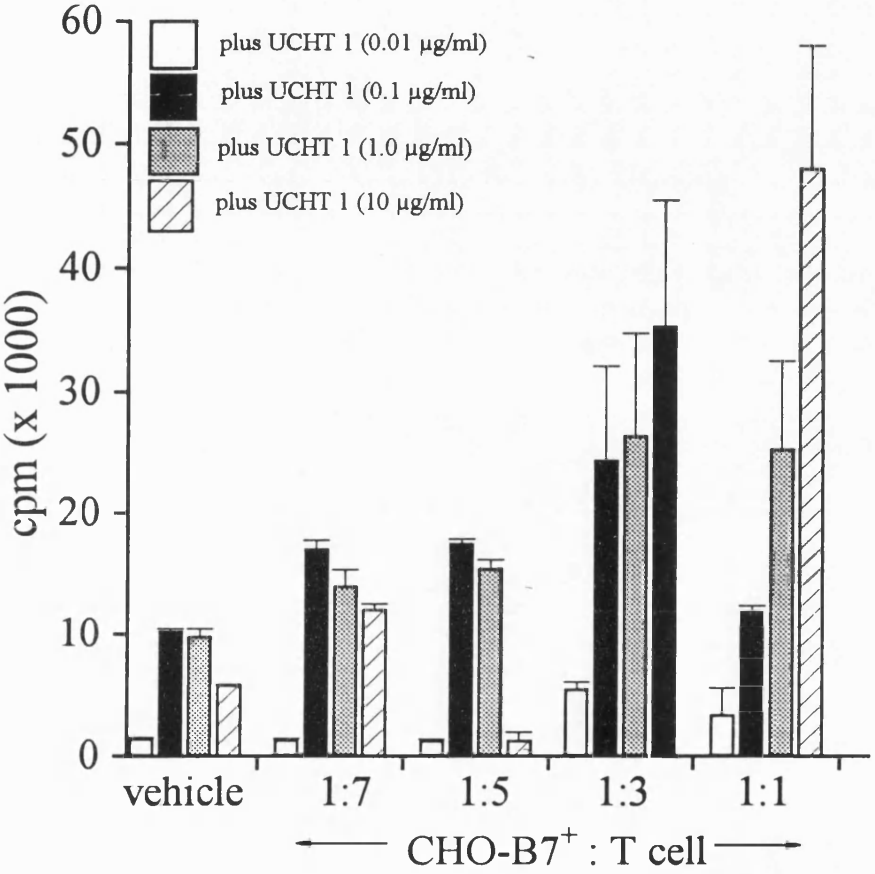


Fig. 3.31 Effect of UCHT1 Plus CHO-B7$^+$ on Proliferation of Primary T Lymphocytes

Effect of 0.01 - 10 µg/ml UCHT1 with CH0-B7$^+$ as APC (CHO-B7$^+$: T cell of 1:7, 1:5, 1:3, 1:1) on proliferation of purified T lymphocytes.

T lymphocytes were purified using a R + D column then treated with UCHT1 and CHO-B7$^+$ for 72 hours. Proliferation was measured by [^{3}H] - thymidine incorporation (cpm x 1000), see Materials and Methods production. Each treatment was repeated in quadruplicate and the mean (± SEM) is shown.

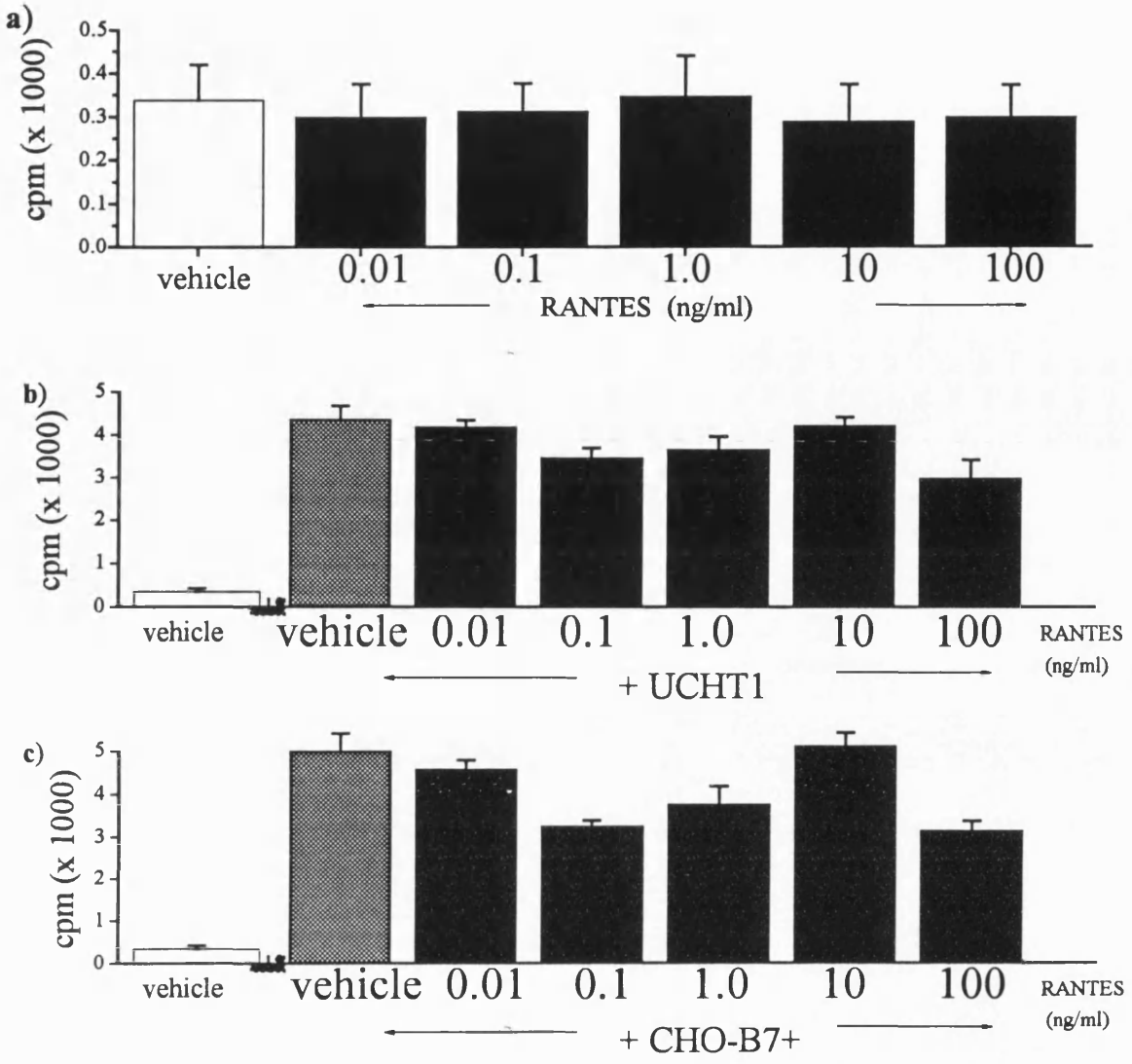


Fig. 3.32 Effect of RANTES on the Proliferation of Primary T Lymphocytes Induced by UCHT1, or CHO-B7$^+$

T lymphocytes were purified using a R+D column then stimulated with **a)** vehicle control (□) or RANTES (0.01 - 100 ng/ml, ■), **b)** vehicle control (□), or UCHT1 (10 μg/ml) in the presence of RANTES (0.01 - 100 ng/ml, ■) or vehicle control (▩), **c)** vehicle control (□), CHO-B7$^+$ (1 : 3 T cells) in the presence of RANTES (0.01 - 100 ng/ml, ■) or vehicle control (▩), for 72 hours. Proliferation was measured by [^{3}H] - thymidine incorporation (cpm x 1000), see Materials and Methods production. Each treatment was repeated in quadruplicate and the mean (± SEM) is shown. Results are from a single representative experiment of three others performed.

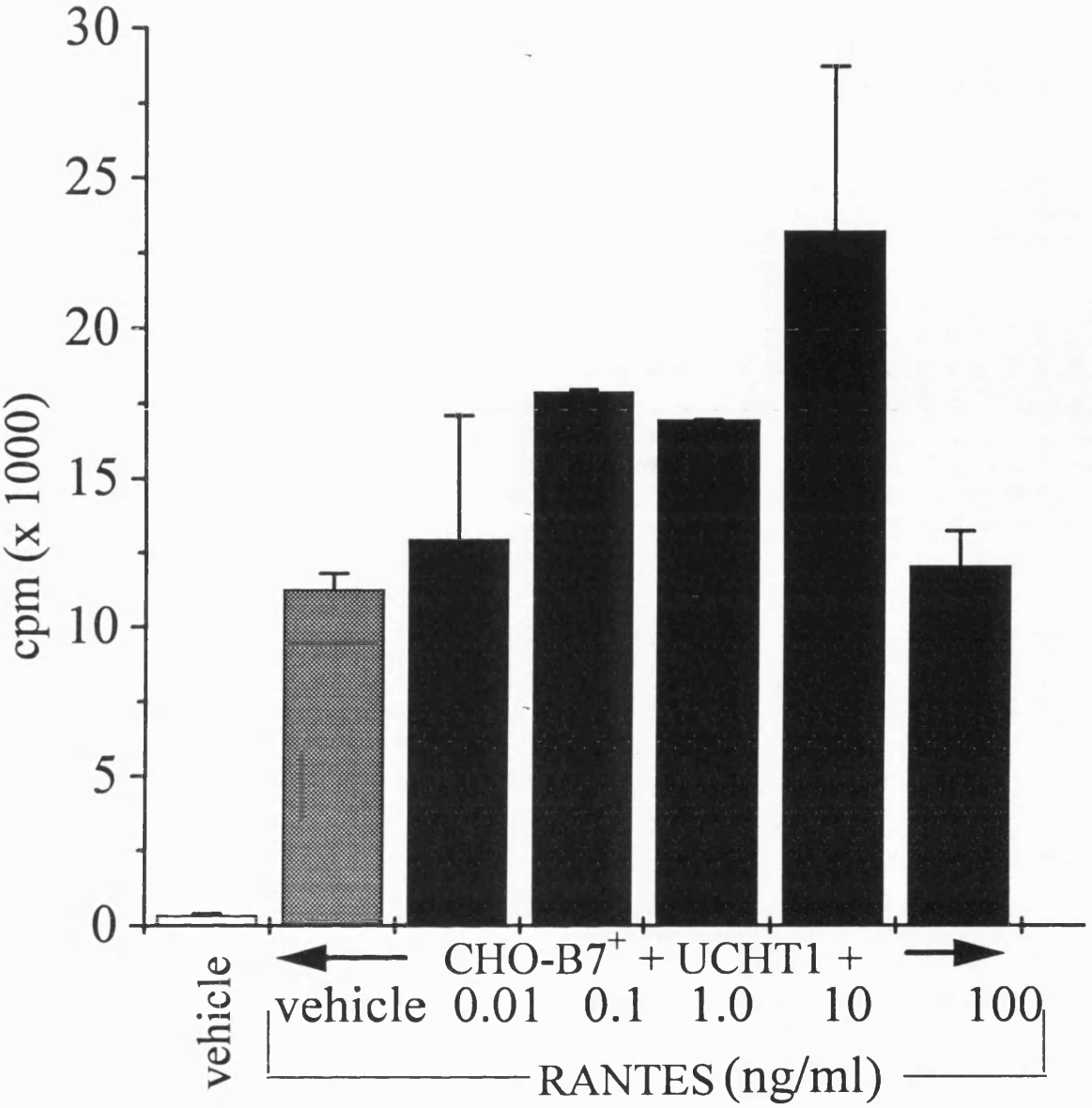


Fig. 3.33 Effect of RANTES on the Proliferation of Primary T Lymphocytes Induced by Costimulation

Effect of RANTES in addition to costimulation of T lymphocytes. T lymphocytes were purified using a R+D column then stimulated with vehicle control, or costimulation with CHO-B7$^+$ (1 CHO-B7$^+$: 3 T cells) and UCHT1 (10 μg/ml) in the presence of RANTES (0.01 - 100 ng/ml, ■) or vehicle control (▩) for 72 hours. Proliferation was measured by [^{3}H] - thymidine incorporation (cpm x 1000), see Materials and Methods production. Each treatment was repeated in quadruplicate and the mean (± SEM) is shown. Results are from a single representative experiment of three others performed.

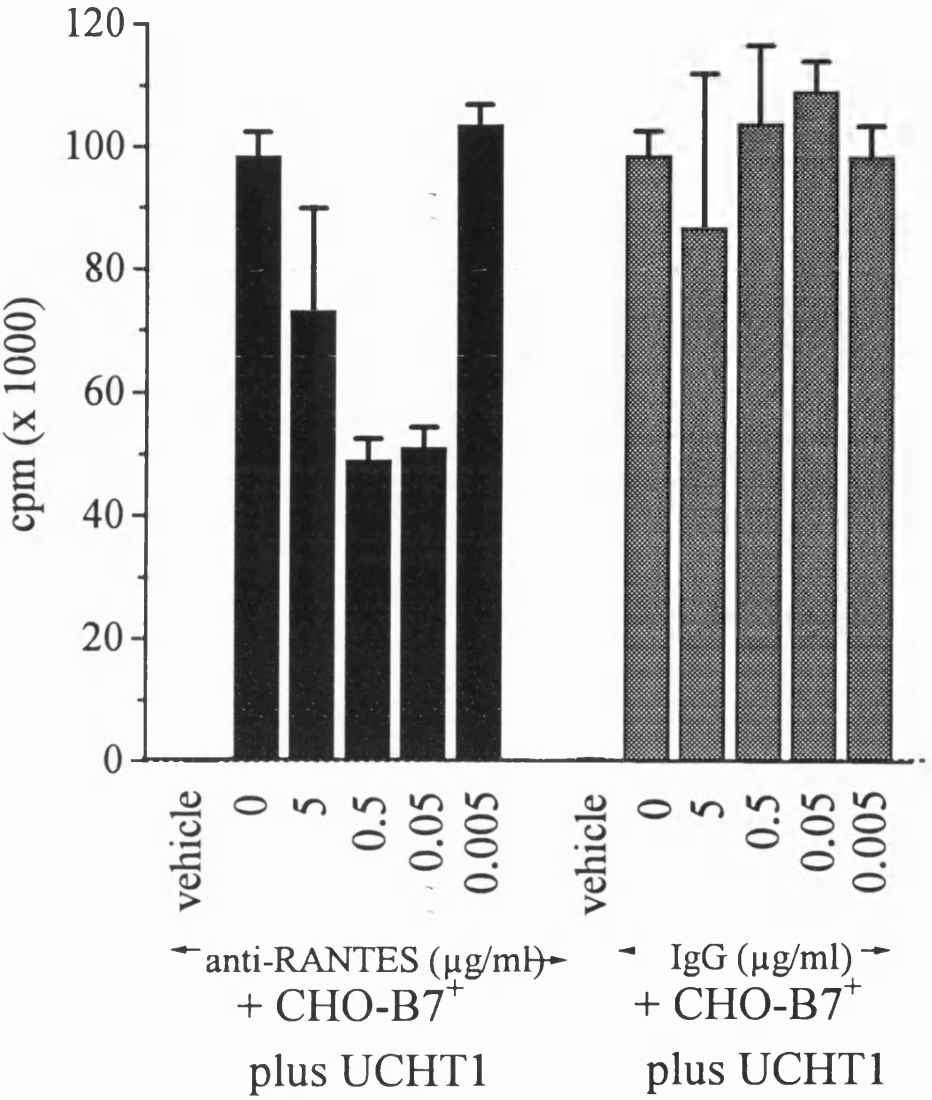


Fig. 3.34 Effect of Anti-RANTES on the Proliferation of Primary T Lymphocytes Induced by Costimulation

Effect of anti-RANTES to costimulation of T lymphocytes. T lymphocytes were purified using a R+D column then stimulated with vehicle control, or costimulation with CHO-B7$^+$ (1 CHO-B7$^+$: 3 T cells) and UCHT1 (10 μg/ml) in the presence of anti-RANTES (5 - 0.005 μg/ml, ■) or IgG control antibody (5 - 0.005 μg/ml, ▩) for 72 hours. Proliferation was measured by [^{3}H] - thymidine incorporation (cpm x 1000), see Materials and Methods production. Each treatment was repeated in quadruplicate and the mean (± SEM) is shown. Results are from a single representative experiment of three others performed.

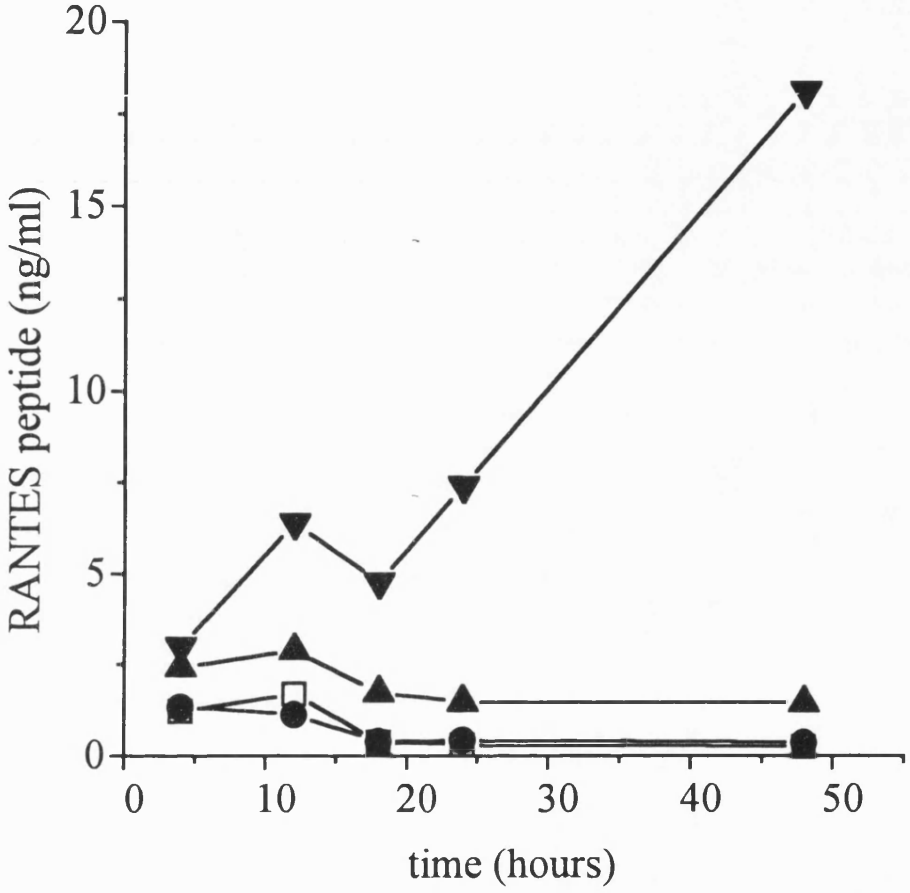


Fig. 3.35 Costimulation of Primary T Lymphocytes Induces RANTES Peptide Production

Costimulation of T lymphocytes with CHO-B7$^+$ and UCHT1 induces RANTES peptide production. T lymphocytes were purified using a R+D column then stimulated with either vehicle (□), UCHT1 (10 μg/ml, ▲), CHO-B7$^+$ (1:3 T cells, ●), or a combination of UCHT1 and CHO-B7$^+$ (▼) for 0 - 48 hours. The RANTES peptide levels were assessed by ELISA, see Material and Methods. Results are from a single representative experiment of three others performed.

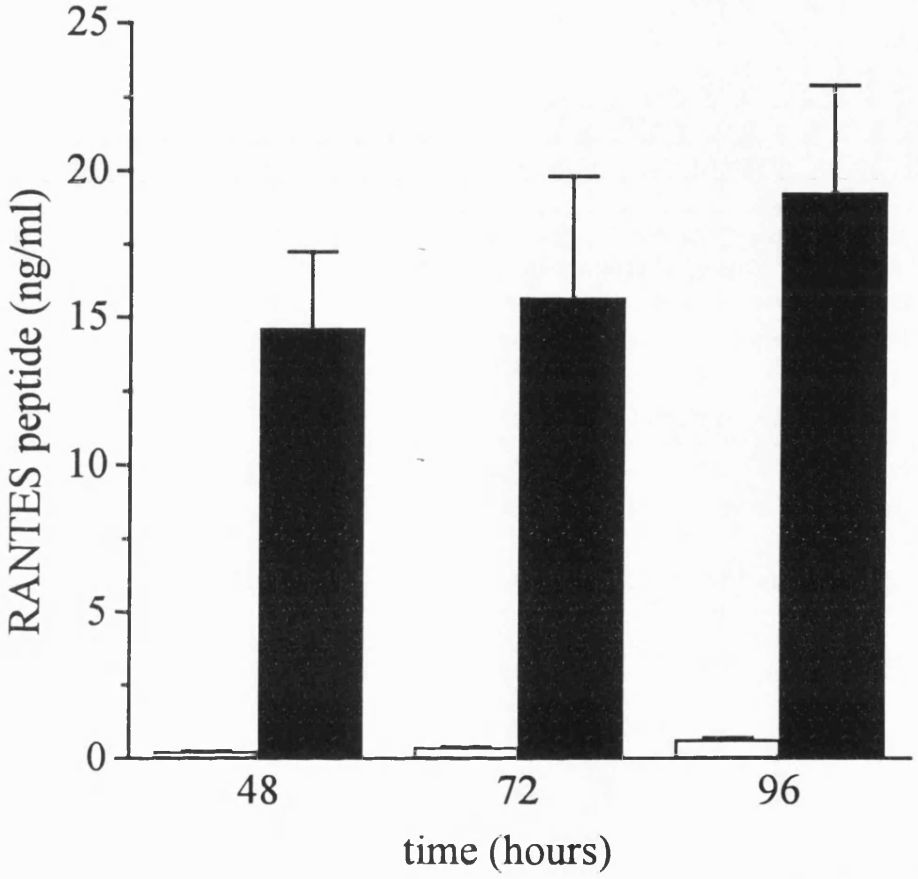


Fig. 3.36 Costimulation of Primary T Lymphocytes Induces RANTES Peptide Production which Plateaus After 96 Hours

T lymphocytes purified using a R+D column were stimulated with either vehicle (□), or UCHT1 (10 μg/ml) and CHO-B7$^+$ (1 : 3 T cells, ■) for 48 - 96 hours. The RANTES peptide levels were assessed by ELISA, see Material and Methods. Each treatment was repeated in triplicate and the means (±SEM) are shown. Results are from a single representative experiment of three others performed.

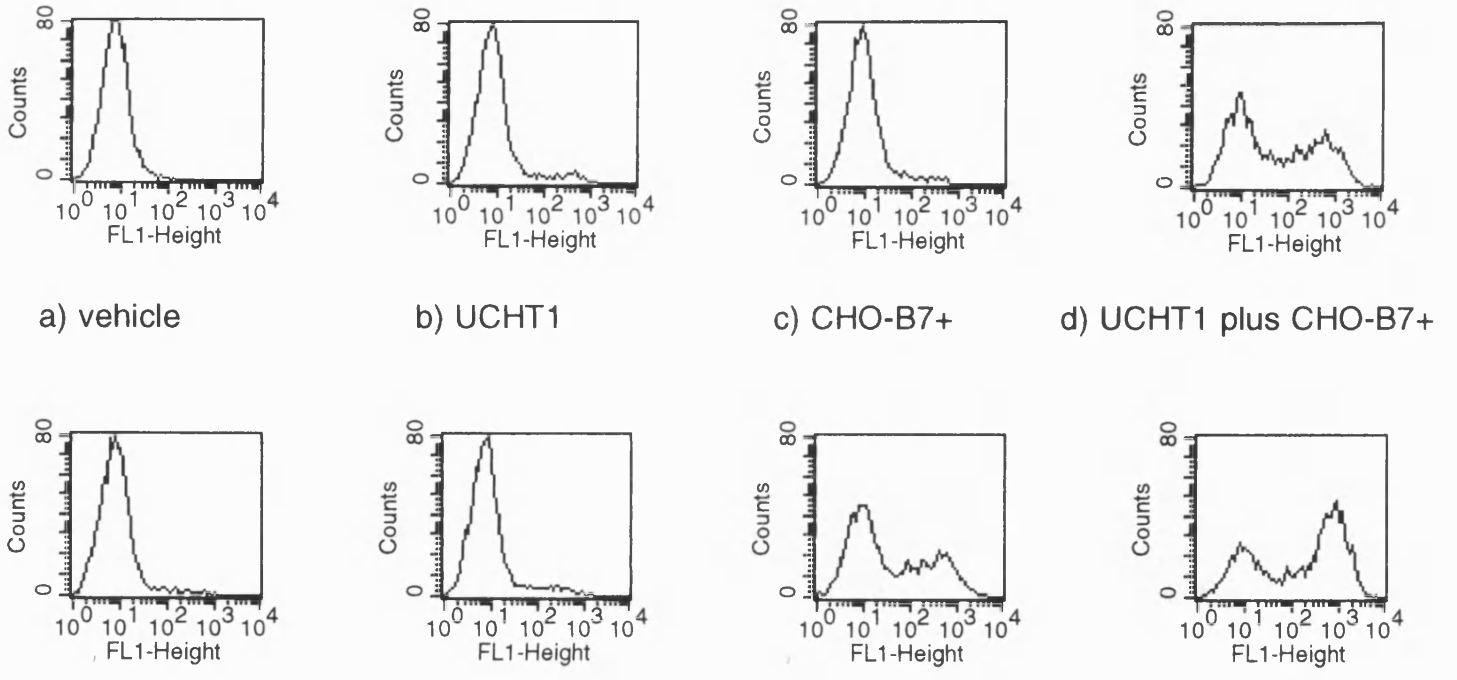


Fig. 3.37 RANTES Modulates the IL-2 Receptor in Primary T Lymphocytes After Costimulation - FACS Traces

T lymphocytes purified using a R+D column were stimulated with either **a)** vehicle, or **b)** UCHT1 (10 μg/ml), or **c)** CHO-B7$^+$, or **d)** costimulation with UCHT1 (10 μg/ml) and CHO-B7$^+$ (1 : 3 T cells). These treatments were repeated in the presence of 1 ng/ml RANTES for 48 hours **e) f) g) h),** respectively. The cells were then stained with FITC-labelled anti-CD25 (anti-IL-2 R), see Material and Methods. Fluorescence intensity was calculated using a FACS vantage, 10 000 viable cells were counted in each event and results were expressed as number of cells against log fluorescence intensity. Results are from a single representative experiment of three others performed.

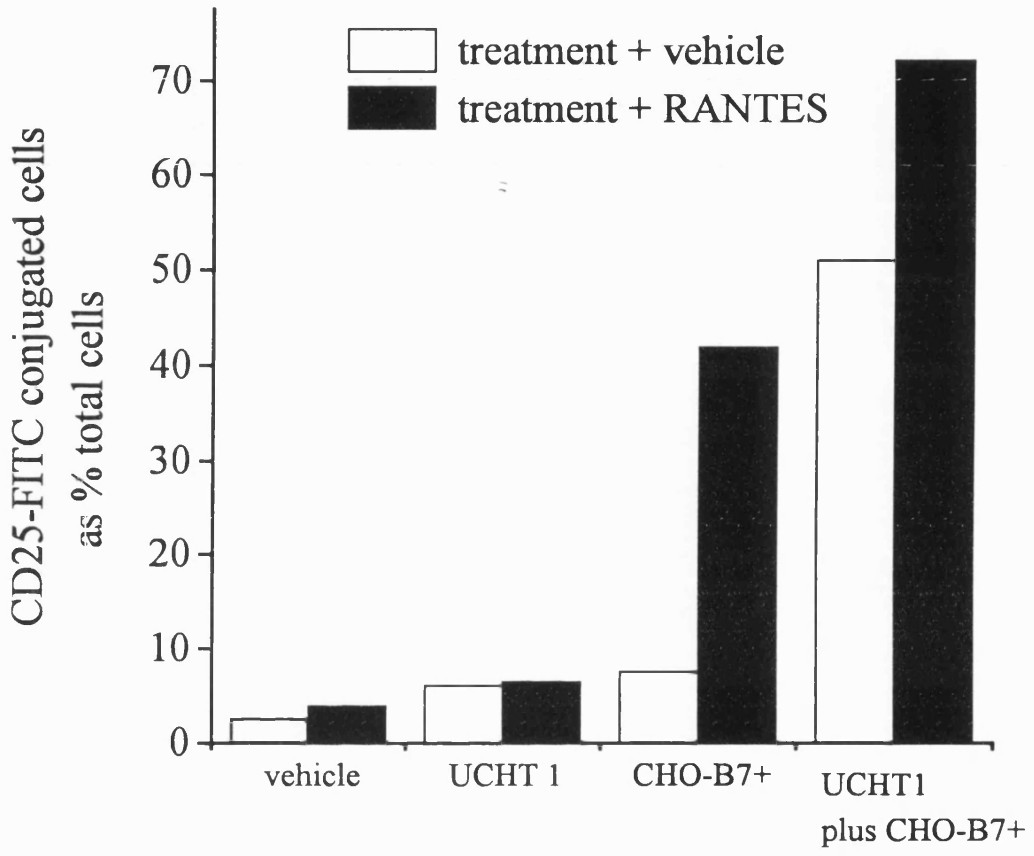


Fig. 3.38 RANTES Modulates the IL-2 Receptor in Primary T Lymphocytes After Costimulation - assessed by FITC-stained cells as a proportion of total cells

T lymphocytes purified using a R+D column were stimulated with either vehicle, or UCHT1 (10 μg/ml), or CHO-B7$^+$, or costimulation with UCHT1 (10 μg/ml) and CHO-B7$^+$ (1 : 3 T cells) in the presence of vehicle (□) or 1 ng/ml RANTES (■) for 48 hours. The cells were then stained with FITC-labelled anti-CD25 (anti-IL-2 R), see Material and Methods. Results are expressed as FITC stained T cells as a proportion of total T cells. Results are from a single representative experiment of three others performed.

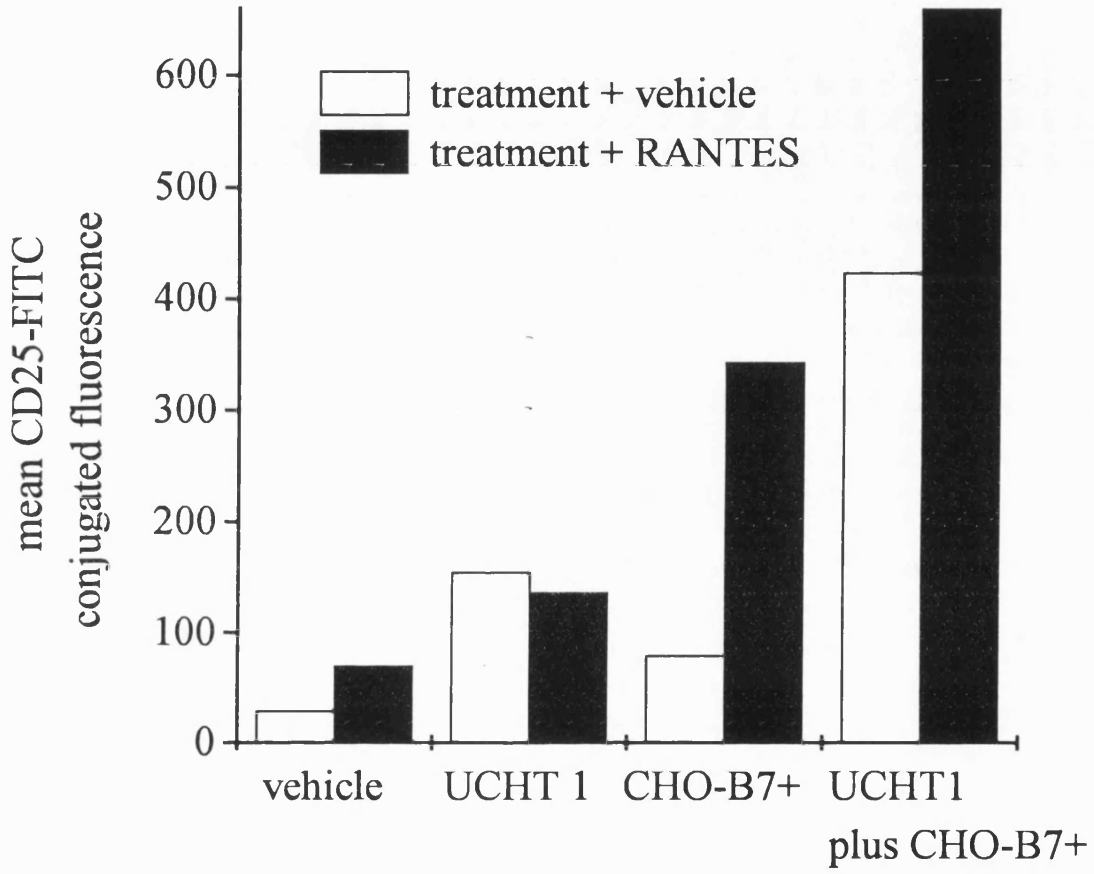


Fig. 3.39 RANTES Modulates the IL-2 Receptor in Primary T Lymphocytes After Costimulation - assessed by mean FITC-fluorescence

T lymphocytes purified using a R + D column were stimulated with either vehicle, or UCHT1 (10 μg/ml), or CHO-B7$^+$, or costimulation with UCHT1 (10 μg/ml) and CHO-B7$^+$ (1 : 3 T cells) in the presence of vehicle (□) or 1 ng/ml RANTES (■) for 48 hours. The cells were then stained with FITC-labelled anti-CD25 (anti-IL-2 R), see Material and Methods. Results are expressed as mean FITC fluorescence and data is from the same samples analysed in **Fig. 3.38**. Results are from a single representative experiment of three others performed.

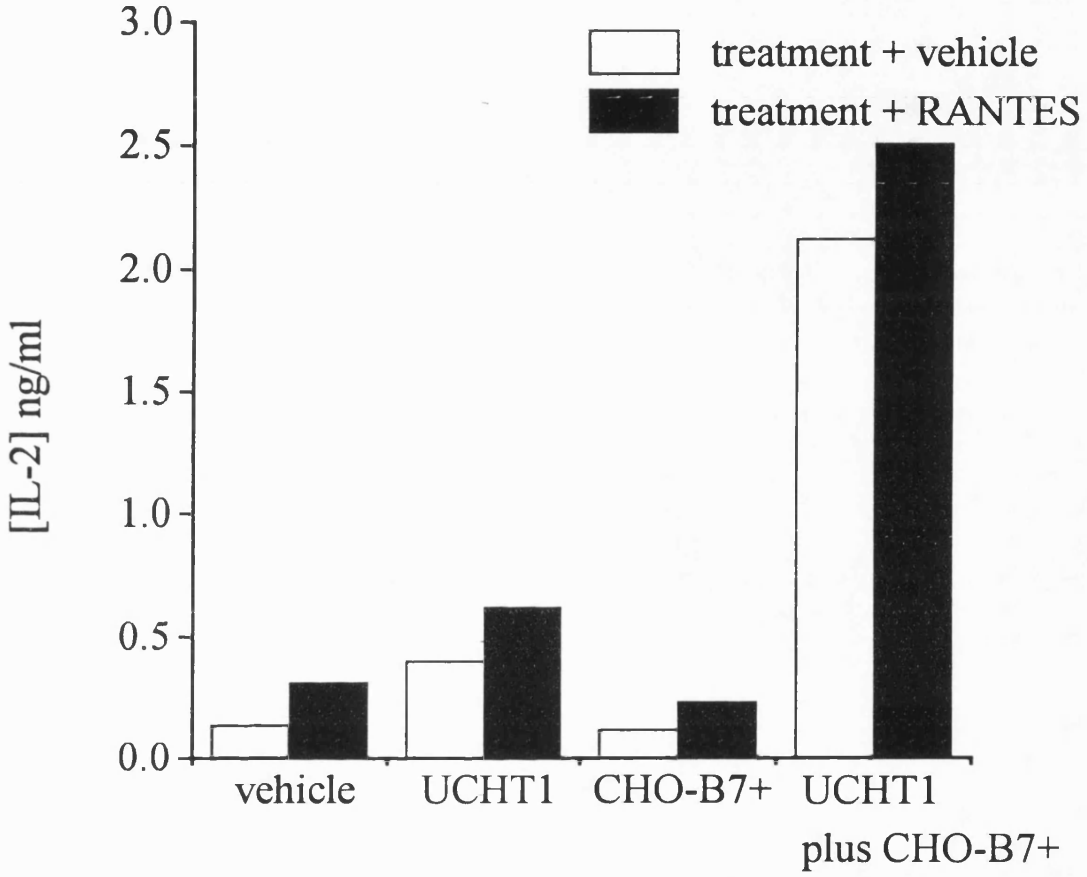


Fig. 3.40 Modulation of IL-2 Peptide Production by RANTES

T lymphocytes purified using a R+D column were stimulated with either vehicle, or UCHT1 (10 μg/ml), or CHO-B7$^+$, or costimulation with UCHT1 (10 μg/ml) and CHO-B7$^+$ (1 : 3 T cells) in the presence of vehicle (□) or 1 ng/ml RANTES (■) for 48 hours. The supernatants were then removes and IL-2 production was assayed using an IL-2 dependent cell line. Results are from a single representative experiment of two others performed.

SECTION FOUR

DISCUSSION

4.1 Migration of T Lymphocytes

4.1.1 Induction of Migration of Primary T Lymphocytes by RANTES

RANTES induces the migration of $CD4^+/CD45RO^+$ cells, memory T lymphocytes *in vitro* (Schall *et al.* 1990). To determine the effects of RANTES on the migration of human T lymphocytes, we performed a series of microchemotaxis chamber assays on primary T lymphocytes, T lymphoblasts and the Jurkat leukaemic T cell line.

RANTES induced migration of primary T lymphocytes that had been purified by either plastic adherence or by using a R+D enrichment column. Either method produced a very high yield of $CD3^+$ cells with few contaminating monocytes, NK cells, or B lymphocytes. The yield, purity, and the extent of migration was not affected by the purification method. The chemotactic effect of RANTES was concentration dependent, with 1 - 10 ng/ml being the optimal concentration, and time dependent. The actual number of cells migrating was largely dependent upon the cell preparation used, with different T lymphocyte populations from different donors having a marked difference in response to the agonists.

It is important to note that leukocyte migration induced by chemicals can be classed as either chemokinesis or chemotaxis (see 3.1.2). Chemokinesis is the change in speed or rate of random turning of cells induced by chemicals in the environment. In comparison, chemotaxis is the directed migration of cells in response to a chemical

stimulus. Most chemotactic agents will also stimulate chemokinesis but it is important to differentiate between the two. This can be done in the 48-well microchemotaxis assay by performing a checkerboard analysis. Chemotaxis involves vectorial migration along a chemoattractant gradient so abolition of this gradient by placing equal concentrations of the chemoattractant in the upper and lower wells of the chamber, will result in decreased migration of the response is chemotactic (Farrar *et al.* 1985). This was the case for the RANTES induced migration, thus RANTES is chemotactic for T lymphocytes, inducing their vectorial migration.

The RANTES induced migration results confirmed what had been shown by previous studies, the optimal RANTES concentration range of 1 - 10 ng/ml and the "bell-shape-like" concentration dependent response curve is consistent throughout the different studies (Schall *et al.* 1990; Murphy *et al.* 1994; Taub *et al.* 1993; Bacon *et al.* 1995). Incubation times are also consistent with 3 - 4 hours causing optimal RANTES induced migration studies. One exception is work by *Schall et al* (Schall *et al.* 1990), where cells were only incubated with RANTES for one hour but an incubation time response curve is not included in this work so it is difficult to ascertain whether they are detecting optimal migration at this incubation time point of one hour.

4.1.2 Induction of Migration of Alternative Sources of T Lymphocytes by RANTES

The same assays were also used on purified T lymphocytes from whole blood packs. RANTES did not induce any migration above vehicle control levels in these T lymphocyte populations. The Blood Transfusion Service will not release whole blood packs until biohazard screening results have been obtained. Therefore, the blood packs

were four days old by the time of purification so the lack of response to RANTES could be explained by the T lymphocytes losing their responsiveness to RANTES after four days in storage. The storage time might have led to downregulation of RANTES receptors, or problems with RANTES binding, or disruption of the normal intracellular signalling pathways. This was unfortunate as the whole blood packs were potentially a good source of large numbers of lymphocytes.

There are two other good sources of large numbers of T lymphocytes : T cell lines, such as the Jurkat cell line and T lymphoblasts, which can be cultured in relatively large amounts. Jurkat T cells did not respond to RANTES in the microchemotaxis assay and no other groups have shown these cells migrating in response to RANTES. The quiescent T lymphoblasts did not respond either to RANTES in this assay. Even at very early stages of T lymphoblast preparation no chemotaxis was observed in response to RANTES, so the response that is seen in primary T lymphocytes is lost at a very early stage even after 3 days PHA stimulation.

The transformation processes that have been performed to produce the Jurkat T cells and T lymphoblasts may be the reason as to why there is no migratory response to RANTES by these cells. The transformation processes may have led to i) the downregulation of the RANTES receptor, ii) disruption or modification of the receptor signalling coupling processes, or iii) disintegration of the full intracellular signalling pathways needed for migration, but as no RANTES receptor has been cloned that is found in T lymphocytes, and the signalling pathways involved in RANTES activation are still not fully identified, this is difficult to investigate (Gao *et al.* 1993; Neote *et al.* 1993).

One of the problems with the work that has been carried out into identifying the RANTES receptor on T lymphocytes is that it has been assumed that this receptor will be G protein linked. The oligonucleotides that have been produced to screen different cDNA libraries and used in Polymerase chain reactions have been based on that assumption (Neote *et al.* 1993). Therefore other oligonucleotides should be made based on amino acid sequences from cytokine receptors that are not G protein linked, but say tyrosine kinase linked such as IL-2 or IFN-α receptor (DeFranco, 1993). These oligonucleotides could then be used as primers in reverse transcription polymerase chain reaction (PCR) using a primary T lymphocyte cDNA library. After molecular cloning and sequencing of the PCR products, new novel clones may be identified. These then could be expressed in cell lines such as human embryonic kidney 293 cells to be able to characterise these receptors fully. In addition, soluble constructs of the C-C CKR1 could be tagged either radioactively or with a fluorescent tag and binding studies could be performed to establish whether this receptor is present on primary T lymphocytes.

The inability of RANTES to induce migration of T lymphoblasts or Jurkat T cells and the lack of chemokine studies performed on T cell lines, that were readily available, indicated that primary T lymphocytes purified from venous blood donations should be used in all the later work.

4.1.3 Other Models of Lymphocyte Migration

The 48-well microchemotaxis assay has several limitations. The membrane is a non-physiological surface for lymphocyte adhesion or migration and all the normal endothelial and extracellular matrix adhesion molecules are absent. More physiological

studies have utilised a transendothelial chemotaxis assay with human umbilical vein endothelial cell monolayers (HUVEC) grown on the membranes (Roth *et al.* 1995). RANTES induced chemotaxis of primary T lymphocytes in this HUVEC migration assay system similarly to the 48-well microchemotaxis assay with preferential migration of $CD45RO^+$ cells. The optimal concentrations for RANTES in these assays were again in the 1 - 10 ng/ml range. Another group has used a human/severe combined immune deficient (SCID) mouse model where SCID mice received human primary T lymphocytes followed by subcutaneous injections of RANTES. RANTES induced migration of lymphocytes, so that lymphocyte accumulation was detected in the skin and tissue from the injection sites (Murphy *et al.* 1994).

4.2 Other Chemotactic Effects of RANTES on T Lymphocytes

Non-motile cells are spherical and the actin filaments present in the cytoskeleton are uniformly distributed. After stimulation of T lymphocytes via TCR coupled mechanisms, there is a large increase in actin polymerisation causing the formation of a dense F-actin collar around the outside of T lymphocytes. This collar is then replaced by the production of enlarged uropods, cytoplasmic projections rich in F-actin, increasing the length of the cell edge, and so polarising the cell shape into that seen in motile cells (Parsey & Lewis, 1993). These uropods are then thought to attach to the substrate, before the cell body moves up over the attachment during migration (Wymann *et al.* 1990).

This is supported by studies using time lapse photography and lymphocyte migration through collagen gels which show that the cells observed to move, were also the ones

that were polarised, and the proportion of cells changing shape was similar to the proportion of cells translocating through the collagen gels (Wilkinson, 1986).

Thus, two other assays were performed to study the chemotactic effects of RANTES in more detail. The effects of RANTES on polarisation, indicating shape change, and the effects of RANTES on actin polymerisation.

4.2.1 Induction of Polarisation of T Lymphocytes by RANTES

RANTES induced a concentration dependent polarisation response in primary T lymphocytes which was also time dependent. Optimal polarisation was seen at 1 - 10 ng/ml RANTES, the same concentrations of peptide that are necessary for maximum migration. This response was seen at 1 hour which is prior to the optimal time for chemotaxis of four hours suggesting that RANTES induces shape change in the T lymphocytes before they migrate. Our results are supported by a study by *del Pozo et al* (del Pozo *et al.* 1995), who not only showed that RANTES can induce polarisation of T lymphocytes, but also caused the redistribution of adhesion molecules such as ICAM-1 and ICAM-3 to the uropod suggesting that RANTES could have an additional role in cell adhesion.

Interestingly it was T lymphoblasts that were used by del Pozo *et al*, illustrating that T lymphoblasts can be responsive to RANTES in polarisation studies but their work did not include migration assays (del Pozo *et al.* 1995). These findings and the inability of RANTES to induce migration of T lymphoblasts would suggest that T lymphoblasts have functioning RANTES receptors and the signalling pathways involved in

polarisation are intact, but migration cannot be induced by RANTES. Thus, a factor that is downstream of T lymphocyte polarisation, but upstream of migration is missing in T lymphoblasts, perhaps the lack of integrins. Integrins, such as $\alpha_4ß_7$, undergo transient interactions with immunoglobulins, such as MAdCAM-1, on the blood vessel walls, which allows for "rolling" of the lymphocytes along the endothelium, which is then followed by an activation signal from chemoattractants causing the firm adhesion between integrin molecules on the lymphocytes and immunoglobulin superfamily ligands on the endothelium, such as LFA-1/ICAM-1 or ICAM-2 or ICAM-3, $\alpha_4ß_7$/MAdCAM-1, and $\alpha_4ß_1$/VCAM interactions, leading to lymphocyte transmigration into the tissues (Ager, 1994).

Polarisation studies were carried out on T lymphocytes purified by the plastic adherence method and using a R+D enrichment column from the same whole blood donation. The method of purification did not affect the extent of polarisation induced by RANTES. Cells that had been purified by plastic adherence compared to those that had been prepared using a R+D enrichment column, did not respond differently in the polarisation assays. This data and the data from the migration assays assured us that when the enrichment column was used for quicker preparation of the cells, the method of preparation was not going to be a factor in the T lymphocyte responses to RANTES.

RANTES specifically induces the migration of $CD45RO^+$ cells, memory T lymphocytes (Schall *et al.* 1990) so the differences in the amplitude of RANTES induced migration and polarisation of different T lymphocyte populations may have been due to the T lymphocyte subsets present in the populations studied. This would be especially valid if there was major variation in the number of $CD45RO^+$ T lymphocytes present.

However, there was no obvious difference in the number of $CD45RO^+$ cells in the populations from five different donors, that could explain the varying responses to RANTES in the polarisation assays. Thus, the $CD45RO^+$ /$CD45RA^+$ ratio variation in the different populations is not likely to be responsible for the different extents of effects induced by RANTES. However, the relatively small proportion of T lymphocytes responding to RANTES would still suggest that it is a subset of T lymphocytes which are RANTES responsive, and the inability to detect significant variation in the number of $CD45RO^+$ T lymphocytes present, may have just been a feature of the lymphocyte populations we examined, or the assay was not sufficiently sensitive to be able to pick up minor variations.

4.2.2 Induction of Actin Polymerisation in T Lymphocytes by RANTES

Actin polymerisation was induced by RANTES in the primary T lymphocytes in a concentration and time dependent manner. Optimal actin polymerisation was detected at 10 minutes, a far earlier optimal time point than that seen for polarisation and chemotaxis induced by RANTES. Thus, actin polymerisation seems to be a much earlier event than either polarisation or migration induced by RANTES. This observed actin polymerisation and resulting shape change may allow the T lymphocytes to more effectively probe the adjacent contact surface for other activating ligands.

Characteristic "bell-shape-like" concentration dependent response curves were observed in all three of the assays. An explanation for this observation, when it was noted by other groups in chemotaxis assays, was that high concentrations of RANTES promoted adherence of the T lymphocytes and hence less locomotion (Bacon *et al.* 1990).

Another possibility is that high concentrations of the chemokine downregulate the RANTES receptor and so reduce the chemotactic effects.

The optimal concentration of RANTES which induced peak responses in the chemotaxis assays, polarisation assays and actin polymerisation assays was constant at 1 - 10 ng/ml. This suggests that these responses are all downstream events of RANTES binding to a similar receptor or receptors that have a similar efficacy. The ability of RANTES to act on resting primary T lymphocytes implies that sufficient high affinity receptor is expressed by these T lymphocytes to enable them to respond to RANTES prior to exposure to antigens.

It had been hoped by Schall *et al*, when they isolated the gene for RANTES, that RANTES would be a specific chemoattractant for T lymphocytes but RANTES is also a chemoattractant for eosinophils, monocytes, basophils and the three receptors that have been identified that bind RANTES, C-C CKR1, C-C CKR2 and C-C CKR4, bind MIP-1α and MCP-1 in addition (Wang *et al.* 1993; Van Riper *et al.* 1994; Combadiere *et al.* 1995a; Power *et al.* 1995). This is analagous to the IL-2R γ-chain which is an indispensable component of the IL-2R, as well as of the IL-4, IL-7 and IL-15 receptors (Taga & Kishimoto, 1995). More recently, a chemokine called lymphotactin has been isolated that specifically regulates both T lymphocyte and B lymphocyte migration. Clearly, lymphotactin has the potential of being an important molecule in the regulation of lymphocyte trafficking. However, the possibility that RANTES causes the selective attraction of T lymphocytes of the memory phenotype, could explain how specific subsets of T lymphocytes are targeted, and may give an indication as to the workings of T cell memory (Schall *et al.* 1990).

4.3 Signalling Pathways and RANTES

The chemoattractant effect of RANTES is well established but the signalling pathways resulting in this effect are not defined. Two pathways were studied in detail: the classical phosphoinositide signalling pathway which involves PLC activation, resulting in PtdIns(4,5)P_2 breakdown and calcium mobilisation and the PI 3-kinase signalling pathway, which causes D-3 phosphoinositide production.

4.3.1 Classical Phosphoinositide Signalling Pathway and RANTES

Evidence has suggested that PLC signalling pathway and the corresponding calcium fluxes are activated by the majority of chemotactic ligands in several systems (Berridge, 1993; Bacon *et al.* 1993). For instance, several groups have shown that C-C chemokines induce calcium fluxes in monocytes (Wang *et al.* 1993), eosinophils (Rot *et al.* 1992; Bourne *et al.* 1995; Kapp *et al.* 1994), and basophils (Bischoff *et al.* 1993). In this study we examined the effcet of RANTES on calcium mobilisation in primary human T lymphocytes. Anti-CD3 (UCHT1) induced a rise in $[Ca^{2+}]_i$ in Jurkat T cells, quiescent T lymphoblast and primary T lymphocytes. This rise has also been shown to correspond with Ins(1,4,5)P_3 formation in Jurkat cells, and indicates the involvement of an intact phospholipase C signalling pathway (Ward & Cantrell, 1988; Imboden & Stobo, 1985). Quiescent T lymphoblasts are readily loaded with fura-2, unlike the primary T lymphocytes. This is seen clearly in the response to UCHT1 which causes a ten fold increase in $[Ca^{2+}]_i$ as compared to the response of primary T lymphocytes to UCHT1 which only induces a three fold increase. There is also a fluctuation in the basal $[Ca^{2+}]_i$ levels in the fura-2 loaded primary T lymphocytes which would indicate that the cells have not loaded properly and that fura-2 is leaking out of the cells and

binding to the extracellular calcium added to the cell suspension. This poor loading may be a reason why nearly all the studies performed on $[Ca^{2+}]_i$ fluxes in T lymphocytes have been performed on T cell lines or T lymphoblasts, not freshly isolated cells (Ward & Cantrell, 1989; Bacon *et al*, 1995).

RANTES (0.1 - 100 ng/ml) did not induce a detectable elevation of basal levels of $[Ca^{2+}]_i$ in primary T lymphocytes, or Jurkat T cells or quiescent T lymphoblasts. The data from the experiments performed on Jurkat T cells and quiescent T lymphoblasts could be explained due to a lack of functioning RANTES receptors, as we could not detect RANTES induced migration in these types of T cell populations either. Chemotaxis control experiments were performed on the same populations of primary T lymphocytes used in these calcium experiments, and RANTES always induced migration of the T lymphocytes, so the lack of calcium mobilisation in response to RANTES is not due to a complete lack of response to RANTES, by the primary T lymphocytes.

Reasons for the lack of a calcium mobilisation response induced by RANTES are unclear. However, it may be that calcium fluxes in specific subsets of the T lymphocyte population cannot be detected using the fluorometric method, especially if the poor loading of fura-2 in the primary T lymphocytes is considered. This possibility is highlighted by the small proportion of T lymphocytes that respond to RANTES in the polarisation assay and the previous studies indicating that only specific subsets of T lymphocytes migrate in response to RANTES. These problems could be overcome by using a more sensitive assay to look at calcium mobilisation in individual cells, not just a cell suspension, such as a FACS (fluorescence activated cell sorter) assay to study

each individual cell, loaded with calcium sensitive dye (Brooks *et al*, 1995). This method would allow cells that exhibit calcium fluxes in response to RANTES, to be selectively sorted and then their phenotype could be assessed. Thus, the RANTES responsive T lymphocyte subsets could be identified.

A recent report by *Bacon et al*, showed RANTES inducing biphasic mobilisation of $[Ca^{2+}]_i$ in T helper cell clones (Bacon *et al.* 1995), which contradicts the findings in this study. Possible reasons for this discrepancy are : i) a different fluorescent $[Ca^{2+}]_i$ indicator was used, indo-1 acetoxymethyl ester, instead of fura -2 which was used in this study, ii) the concentration of RANTES used to stimulate the T cells were 10 - 100 fold higher concentrations than that produce optimal migration, although there is no indication of what concentration of RANTES is seen at the receptor, especially as RANTES may exist as a dimer in solution (see 1.2.1), and iii) the responses were only seen in a T helper cell line not in Jurkat T cells. *Loetscher et al*, also, have reported calcium fluxes after RANTES stimulation of $CD4^+$ and $CD8^+$ T cell clones. However the responses observed were only two fold increases above basal $[Ca^{2+}]_i$ and the work was not repeated in primary T lymphocytes (Loetscher *et al.* 1994). The data presented in this study, however, indicates that chemotaxis of T lymphocytes, as determined under the *in vitro* conditions described, is not mediated by significant changes in intracellular calcium levels and assocaited downstream effector events.

Activation of quiescent T lymphoblasts and primary T lymphocytes by UCHT1 increased the levels of phosphatidic acid (PtdOH), a product of diacylglycerol metabolism and therefore an alternative indicator of PLC activation. However, PtdOH can also be a product of PLD activation, so $PtdIns(4,5)P_2$ levels were examined, in

addition, to indicate PLC activation. PtdIns(4,5)P_2 levels in the quiescent T lymphoblasts and primary T lymphocytes did appear to be reduced after UCHT1 stimulation. This supports the PtdOH findings and demonstrates that anti-CD3 stimulation results in activation of the phospholipase C signalling pathway. However, RANTES stimulation, did not alter the levels of either PtdOH or PtdIns(4,5,)P_2 in either of the cell populations. This evidence confirms the results from the calcium determination experiments which indicated that the phospholipase C signalling pathway is not involved in RANTES stimulation of T lymphocytes.

4.3.2 PI 3-Kinase Signalling Pathway and RANTES

In mammalian cells, PI 3-kinase has been shown to associate, after receptor activation, directly or indirectly with numerous growth factors, including the IL-4 receptor and IL-2 receptor (Izuhara & Harada, 1993) and is coupled to CD28 (Ward *et al.* 1993; Pages *et al.* 1994; Truitt *et al.* 1994, Prasad *et al.* 1994), CD3 (Exley *et al.* 1994), CD4 (Thompson *et al.* 1992) T cell antigens. The D-3 phosphoinositide lipids, particularly PtdIns(3,4,5)P_3 generated by PI 3-kinase activity, have been postulated to act as second messenger molecules and implicated in the regulation of cell growth and proliferation.

The activation of PI 3-kinase by a specific receptor should lead to the accumulation of D-3 phosphoinositides such as PtdIns(3,4,5)P_3. These lipids are generally measured after metabolic labelling of cells with [^{32}P]-orthophosphate, as described in Materials and Methods. However, D-3 phosphoinositides form only a minor fraction of the total lipid content, and detection of D-3 phosphoinositide accumulation following receptor ligation, *in vivo*, requires substantial cell numbers and adequate radiolabelling of the

phospholipids with [^{32}P]-orthophosphate.

We were unable to generate sufficient primary T lymphocyte numbers from freshly purified blood to enable the detection of D-3 phosphoinositides at a satisfactory level, as the radiolabelling of the resting primary cells was poor. For instance, the efficiency of radiolabelling was assessed by examining the number of cpm in the PtdIns pool. Thus, the radioactivity in the PtdIns pool of primary T lymphocytes was in the range of 0.8 - 1.7 x 10^4 cpm, compared to the radioactivity in quiescent T lymphoblasts, which was in the range of 8 - 20 x 10^4 cpm . Thus, radiolabelling of quiescent T lymphoblasts was not a problem, but RANTES did not increase D-3 phosphoinositide accumulation. Again, this lack of response to RANTES could be due to the inability of T lymphoblasts to bind RANTES, not necessarily because RANTES does not activate PI 3-kinase, although the work by *del Pozo et al*, indicates that T lymphoblasts may have RANTES receptors (del Pozo *et al.* 1995). However, this group did not examine the PI 3-kinase signalling pathways.

RANTES did induce PI 3-kinase activity in primary T lymphocytes *in vitro* as assessed by : i) the increase in lipid kinase activity in anti-p85 subunit immunoprecipitates, ii) the inhibition by wortmannin, a specific PI 3-kinase inhibitor, of the RANTES induced chemotaxis, polarisation and actin polymerisation, and iii) the inhibition by wortmannin of the RANTES induced lipid kinase activity seen in anti-p85 subunit immunoprecipitates.

Howver, some important control experiments should be performed to clarify these results. Firstly, the lipid product of the anti-p85 subunit immunoprecipitates should be

analysed by quantitative HPLC to check that it is 3-phosphorylated lipids that have been produced not 4-phosphorylated lipids, confirming that the anti-p85 immunoprecipitated PI 3-kinase activity. This however may not be possible as only low levels of radiolabelled product were obtained. Alternatively, the immunoprecipitates could be examined for their sensitivity to adenosine (300 μM) and detergents such as NP40 (0.5 %). PI 3-kinase is inhibited in the presence of detergent but not affected by adenosine, whereas PI 4-kinase is inhibited by adenosine but not affected in the presence of detergents (Cochet *et al.* 1991, Ward *et al.* 1992b). Western blot analysis could also be used to analyse the quantity of protein being immunoprecipitated, to ensure that the observed changes in PI 3-kinase activity are not just a result of differences in quantities of protein.

As the peak induction of PI 3-kinase activity occurs at 10 minutes post-RANTES and the maximum actin polymerisation occurs at a similar time point, this implies a significant role for a PI 3-kinase in actin polymerisation of primary T lymphocytes. In comparison, chemotaxis and polarisation occur at much later time points, suggesting the involvement of other signalling mechanisms distal to PI 3-kinase. However, the marked inhibitory effect of wortmannin on polarisation and chemotaxis indicates a key role for PI 3-kinase.

Our studies also support the existence of several PI 3-kinase isoforms with different sensitivities to wortmannin which are activated *in vivo* by RANTES, as the functional effects of chemotaxis including migration, polarisation and actin polymerisation are more sensitive to wortmannin inhibition, with an IC_{50} of between 0.04 - 0.5 nM, than the *in vitro* kinase activity upregulated by RANTES, which wortmannin inhibits with

an IC_{50} in the range of 10 - 100 nM.

PI 3-kinase activation has been implicated in cell growth and proliferation, sorting and trafficking of proteins in eukaryotic cells (Volinia *et al.* 1995), and actin polymerisation. The role of PI 3-kinase activation in protein trafficking may be particularly relevant to migration, as these responses require substantial cytoskeletal reorganisation. In addition, D-3 phosphoinositides can activate PKC isoenzymes such as PKCδ, ϵ, ζ and η. *Phatak et al* have shown that PKC activation is important in actin polymerisation. Engagement of the TCR by anti-CD3 causes a rapid increase in actin polymerisation in T lymphocytes but the concomitant rise in $[Ca^{2+}]_i$ was not necessary nor sufficient for the F-actin increase, as calcium ionophores caused a similar rise in $[Ca^{2+}]_i$ but had no effect on F-actin (Phatak & Packman, 1994). However, inhibitors of PKC lowered the resting cellular F-actin and partially blocked the increase in F-actin caused by anti-CD3 but had no effect on $[Ca^{2+}]_i$, indicating a role for PKC in actin polymerisation, independent of calcium mobilisation. One of the major intracellular PKC substrates, PKC substrate protein, cross links F-actin when dephosphorylated and is located at site of membrane and microfilament interactions (Toker *et al.* 1994). This could explain the requirement for intact PI 3-kinase signalling in migration and actin polymerisation of T lymphocytes and the inability to detect calcium mobilisation. Several other groups have implicated PI 3-kinase to be involved in the regulation of actin polymerisation and chemotaxis. For instance, FMLP stimulation of human neutrophil locomotion occurs with an increase in PI 3-kinase activity (Okada *et al.* 1994), and platelet derived growth factor mediated actin rearrangements in fibroblasts are sensitive to nanomolar concentrations of wortmannin (Wennstrom *et al.* 1994).

The recent work indicating the involvement of the Ras GTPases downstream of PI 3-kinase (see introduction) would also suggest a role for PI 3-kinase activation in cytoskeletal reorganisation. In fibroblasts, actin polymerisation is controlled by the Rho GTPase family. Filamentous cytoplasmic projections, referred to as filipodia are controlled by Cdc42 (Nobes & Hall 1995), Rac regulates protusions kown as lamellipodia which are though to have a role in cell migration and Rho controls the formation of stress fibres (Symons 1996). In T lymphocytes, Cdc42 has been shown to regulate cellular polarisation detected after antigenic stimulation (Stowers *et al.* 1995). Rho can be inhibited by C3 transferase which inactivates the GTPase by specific ADP-ribosylation, therefore this transferase could be used as a tool to identify whether Rho is a downstream target of RANTES induced PI 3-kinase (Fry *et al.* 1994). Once the T lymphocyte RANTES receptor has been identifed and expressed in a convenient cell line, overexpression of the various GTPases and mutations could be performed to try to identify which, if any, GTPases are involved in RANTES induced chemotactic effects.

RANTES induces increases in lipid kinase activity in anti-p85 subunit immunoprecipitates. p85 is a component of the PTK/SH2 linked isoform of PI 3-kinase However, the receptors that have been identifed for RANTES are G protein coupled (Van Riper *et al.* 1993; Wang *et al.* 1993), although none of these receptors have been detected on T lymphocytes. This could be explained by the existence of another RANTES receptor, specific for T lymphocytes, that is protein tyrosine kinase coupled. To support this, *Bacon et al* have shown that RANTES acts through two pharmacologically distinct signalling pathways, one linked to a PTX-sensitive G protein and one to PTK activation which can be inhibited by herbimycin A, a selective inhibitor

of Src family PTK (Bacon *et al.*, 1995). In addition, these pathways appear to be independent, because each remained unaltered when the other was fully inhibited. However, another possibility is that the RANTES/G protein coupled receptor may be activating a PTK/SH2 coupled PI 3-kinase further downstream. Anti-p85 monoclonal antibody can remove the activity of G protein coupled PI 3-kinases, in human platelt cytosol, suggesting that this activity must, in addition, involve the PTK/SH2 isoform of PI 3-kinase (Downes *et al.*, 1994).

One area that has not been covered is how RANTES binding to its putative receptor could bring about these effects on PI 3-kinase. The regulation of PI 3-kinase is still unclear and is complex. It may involve conformational changes in the subunits, translocation of the enzyme from the cytosol to adjaecent to the cell membrane where the lipid substrates are found, or phosphorylation of the PI 3-kinase subunits. Several models have been proposed : e.g. i) direct interaction with protein tyrosine kinase receptors (e.g. PDGF receptor), stimulation of the transmembrane receptor causes activation of its kinase domain and autophosphorylation, enabling PI 3-kinase to bind via its SH2 domains and thus being directly activated, or through binding via SH3 domains on *Src* kinases (Otsu *et al.* 1991; Pleimann *et al.* 1994), ii) activation via the G protein ßgamma subunit (Stephens *et al.* 1994), iii) more recently, experimental evidence has suggested that that Ras may act upstream of PI 3-kinase. Ligand bound receptors promote the active GTP- bound form of Ras, by enhancing the ability of guanine nucleotide exchange factor to accelerate the change from GDP to GTP, then Ras may act as a chaperone to ensure lipids and enzyme interact in activated cells (Feig *et al.* 1994) PI 3-kinase activity has been found in association with Ras (Fry *et al.* 1994) but clarifying whther Ras is acting upstream or downstream of PI 3-kinase has

been very difficult. Rho may also be involved, as the p85 subunit has a rhoGAP domain, and Rho function has been shown to be necessary for PI 3-kinase activation.

To identify whether translocation is important in RANTES activation of PI 3-kinase, not only should total cellular activity of PI 3-kinase be examined but in addition, the cells should be fractionated. PI 3-kinase activity can then be measured in the the subcellular fractions. If the activity of the enzyme in the membrane fraction has increased and the activity in the soluble fraction decreased, then translocation is involved in the RANTES induced activity of PI 3-kinase (Susa *et al.* 1992). Conformational changes could be detected by examining the crystal structures of the PI 3-kinase before and after activation by RANTES (Waksman *et al.* 1993)

The PTK inhibitor, herbimycin, could be used to investigate the involvement of PTK in RANTES activation of PI 3-kinase (Bacon *et al.* 1995). Anti-phosphotyrosine immunoprecipitates could be performed on RANTES stimulated cells and examined to see if they contain PI 3-kinase subunits by Western blotting with anti-p85. This would indicate whether RANTES is regulating PI 3-kinase activity by a tyrosine kinase mechanism or not (Ward *et al.* 1992b).

Pertussis toxin, an inhibitor of G proteins, or mastoparan (a peptide from wasp venom which activates certain G proteins) could be used to see if there is any involvement of G proteins in the RANTES induction of PI 3-kinase activity (Bacon *et al.* 1995; Weingarten *et al.* 1990). Immunoprecipitates with anitbodies against the Ras proteins could be carried out to see if PI 3-kinase activity could be co-immmunoprecipitated. The involvement of Rho could be clarified by using the enzyme C3 transferase which

inhibits Rho by specific ADP-ribosylation (Fry *et al.* 1994).

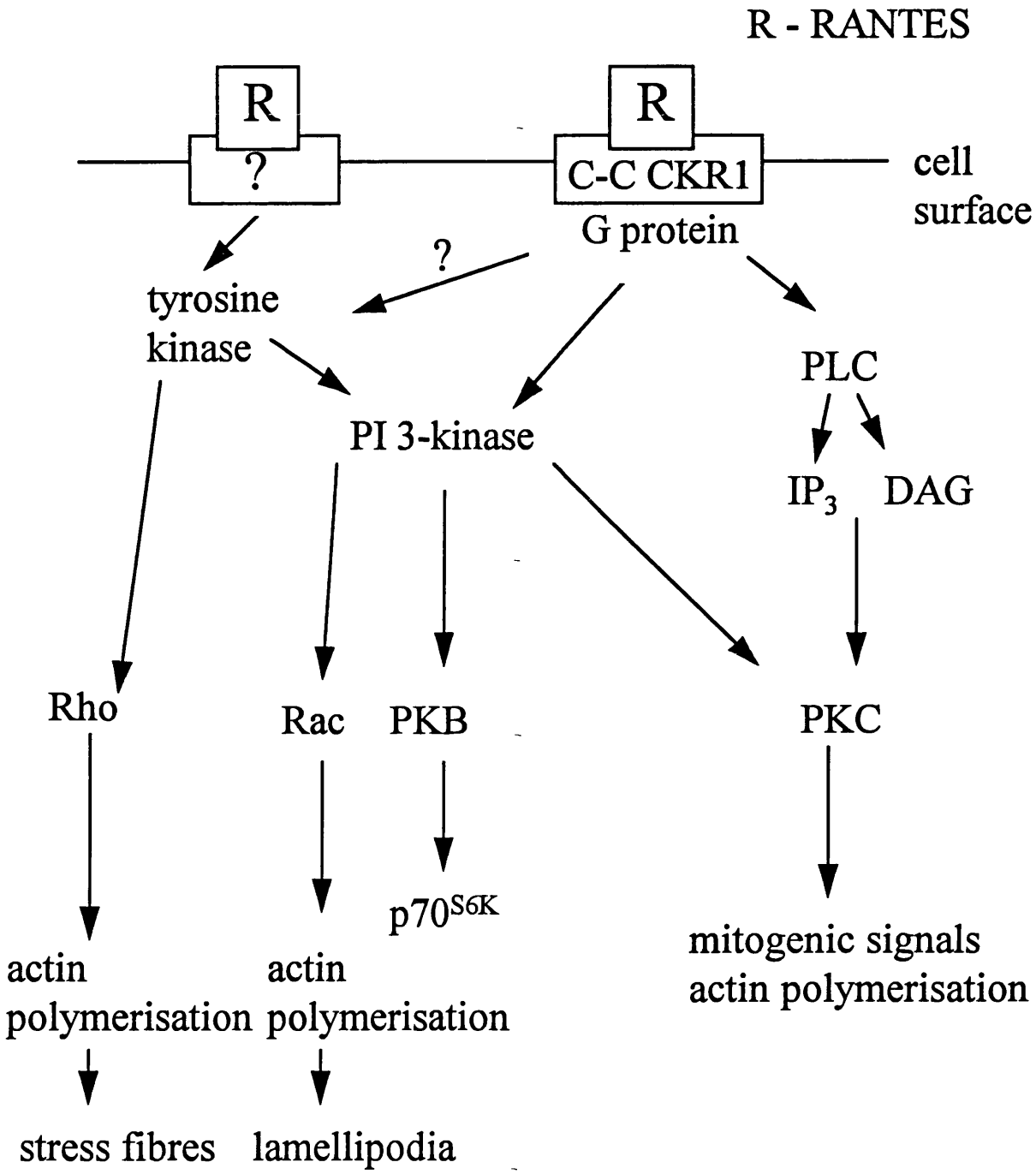


Fig. 4.1 Model of RANTES Signalling in T Lymphocytes

4.4 Costimulation and RANTES

Two signals are required for complete activation of T lymphocytes. Engagement of the TCR by Ag-MHC is not sufficient for IL-2 production and proliferation, and a second costimulatory signal is required (Turner *et al.* 1995). The major costimulatory signal identified is provided by the interaction of the CD28 cell surface ligand with the B7 family on the antigen presenting cells. PI 3-kinase has been shown to be critical in CD28 costimulation in some T lymphocyte models (Sansom *et al.* 1995; Ward *et al.* 1993) with abrogation of the generation of D-3 phosphoinositides resulting from CD28 activation, preventing CD28 mediated costimulation (Ward *et al.* 1995). RANTES activates PI 3-kinase. PI 3-kinase activation has been demonstrated to be a key event in CD28-mediated costimulatory events. This suggested that RANTES activation of PI 3-kinase may also provide a costimulatory signal in T lymphocyte activation. Indeed our results show that given specific costimulatory conditions, RANTES could modulate T lymphocyte proliferation.

It has previously been shown that RANTES at high concentrations of 0.1 - 10 μg/ml can cause proliferation of a T helper cell line, but these concentrations do not cause optimal migration of T lymphocytes *in vitro* (Bacon *et al.* 1995). This study found that RANTES alone was ineffective in causing proliferation of primary T lymphocytes, at concentrations of 0.1 - 100 ng/ml *in vitro*, and RANTES, in combination with UCHT1 alone or CHO-B7$^+$ alone, did not increase the levels of proliferation above those seen with UCHT1 alone or CHO-B7$^+$ alone. However, when RANTES was added in combination with the two costimulatory factors, UCHT1 and CHO-B7$^+$, it potentiated the effects seen with costimulation alone, in a concentration dependent manner, increasing proliferation by a factor of 2.1 $\pm$ 0.3 (n = 3).

Anti-RANTES inhibits the proliferation of the T lymphocytes seen in the presence of the costimulatory factors, UCHT1 and CHO-B7$^+$. Moreover, these inhibitory effects seen in the presence of decreasing concentration of anti-RANTES, produce a curve that is the inverse to the "bell-shape-like" concentration dependent response curve characteristically generated in response to RANTES stimulation, in assays such as chemotaxis, polarisation and actin polymerisation. High concentrations of anti-RANTES (e.g. 5 μg/ml) and low concentrations of anti-RANTES (e.g. 0.005 μg/ml) have only a fraction of the inhibitory capacity that intermediate concentrations of anti-RANTES such as 0.5 μg/ml have. Thus, this highlights the fact that high concentration of RANTES may induce downregulation of the RANTES effects observed, which may be due to increased T lymphocyte adherence or downregulation of receptors.

Anti-RANTES inhibits proliferation of costimulated T lymphocytes suggesting that RANTES is produced endogenously during costimulation of primary T lymphocytes. Large amounts of RANTES peptide (up to 25 ng/ml) are produced when primary T lymphocytes are costimulated with UCHT1 and CD28. This is in agreement with previous reports showing production of many lymphokines after T lymphocyte costimulation including IL-2, IL-3, IL-4, IL-5, IL-6, TNF - α and IFN - γ (Fraser *et al.* 1993). Recently, *Ortiz et al*, have studied the transcription factors regulating the RANTES chemokine gene. AP-1, NFκB, CD28RE, NFAT-1 and Rel-1 binding sites have been detected in the immediate upstream region of the RANTES gene in T cell lines (Ortiz *et al.* 1996; Nelson *et al.* 1993). CD28RE is a CD28 response element that has also shown to be located on the IL-2 promoter, and CD28 is thought to regulate IL-2 expression through this binding site . Therefore, CD28 could regulate the RANTES promoter in a similar manner. Indirect evidence also suggests that CD28 costimulation

may regulate some of the other transcription factors involved in controlling the RANTES promoter, including c-Rel and NFκB via $p70^{S6}$ kinase, and AP-1 via JNK activation (Fraser *et al.* 1992; Rayter *et al.* 1992; Rincon *et al.* 1994).

Purified subsets of T lymphocytes have also been examined for RANTES peptide secretion and mRNA expression (Conlon *et al.* 1995). An antibody against the TCR, or phorbol myristate acetate (PMA) in combination with ionomycin, were used to activate human peripheral blood lymphocyte subsets. Low constitutive expression of RANTES was observed in all the T lymphocyte subsets, but after activation (18 hours), chemokine expression increased and indicated that $CD8^+$, $CD45RO^+$ T lymphocytes were the dominant source of RANTES. $CD8^+$ cells are considered primarily as cytolytic cells and have been judged to have little secretory ability. These RANTES results would suggest that they also have a helper function and give RANTES another role in T lymphocyte function.

IL-2 receptor upregulation and IL-2 production are indicators of T lymphocyte activation. We were unable to detect any consistent induction of IL-2 receptor upregulation or IL-2 production when primary T lymphocytes were stimulated with RANTES alone. However, RANTES did potentiate IL-2 receptor upregulation seen with CHO-$B7^+$ and UCHT1 in combination with CHO-$B7^+$. This did not correlate with the effects on CHO-$B7^+$ dependent proliferation, as RANTES did not cause any potentiation of proliferation seen with CHO-$B7^+$ alone. This disparity suggests that different RANTES signalling pathways may be involved in IL-2R upregulation and proliferation.

IL-2 production was not affected significantly by RANTES stimulation in combination with either UCHT1 alone or CHO-B7$^+$ alone. Furthermore, RANTES in combination with both the costimulatory factors did not upregulate IL-2 production. Therefore, RANTES appears to upregulate IL-2R expression without any significant effects on IL-2 production. CD28 costimulation upregulates IL-2R α-chain (Cerdan *et al.*, 1992), ß-chain (Cerdan *et al.*, 1995) and γ-chain (personal communication with S.G. Ward). In this study RANTES modulates expression of the IL-2R α-chain but it could also regulate expression of the α- and γ-chains. RANTES signalling pathways, however, do not appear to be coupled to IL-2 production.

IL-2R α-chain is expressed after mitogenic or antigen stimulation of human T lymphocytes (Leonard *et al*, 1985). Expression of the α-chain gene is regulated by transcriptional activation and post-transcriptionally by mRNA stabilisation (Leonard *et al.* 1985; Cerdan *et al.* 1992). The transcription factor, NFkappaB, has been suggested to play a role in the regulation of the α-chain transcription in primary T cells (Lowenthal *et al.* 1989) and experiments have been carried out to show that the nuclear amount of the NF-kappaB complex directly correlates with IL-2R α-chain expression (Pimentel-Munos *et al.* 1994). Tumour necrosis factor-α and/or IL-2 in T lymphocytes activated by anti-CD3 upregulates the amount of IL-2R α-chain mRNA and surface expression of the α-chain was also increased (Pimentel-Muinos *et al.* 1994). More recently, work has shown that upregulation of the IL-2R ß-chain is also controlled through combined transcriptional and post-transcriptional mechanisms (Cerdan *et al.* 1995). CD2 and CD28 costimulation led to an increase in transcription rate and transcript stabilisation of the ß-chain, detected by treating with actinomycin D.

The pathways involved in RANTES induced potentiation of the costimulatory response have not been identified. The findings in this study would indicate that PI 3-kinase is pivotal to RANTES activation of T lymphocytes, suggesting that this pathway may be involved in RANTES induced proliferation. However, CD28 activation is also linked to PI 3-kinase activity, so why activation of this enzyme by RANTES potentiates costimulation is not fully understood. There are several possible explanations including the existence of multiple PI 3-kinase isoforms with specific substrates and effector molecules activated by RANTES or CD28. Alternatively, the different receptors may couple in different ways to the same isoform of PI 3-kinase and act additively.

A possible model to explain the findings in this study is that CD28 costimulation of T lymphocytes not only increases IL-2 production but also causes an increase in RANTES production. RANTES released from the cells, could act in an autocrine manner on RANTES cell surface receptors on these cells, and in a paracrine manner on other T lymphocytes, to amplify the costimulatory response, resulting in increased proliferation and IL-2 receptor upregulation (**Fig. 4.2**).

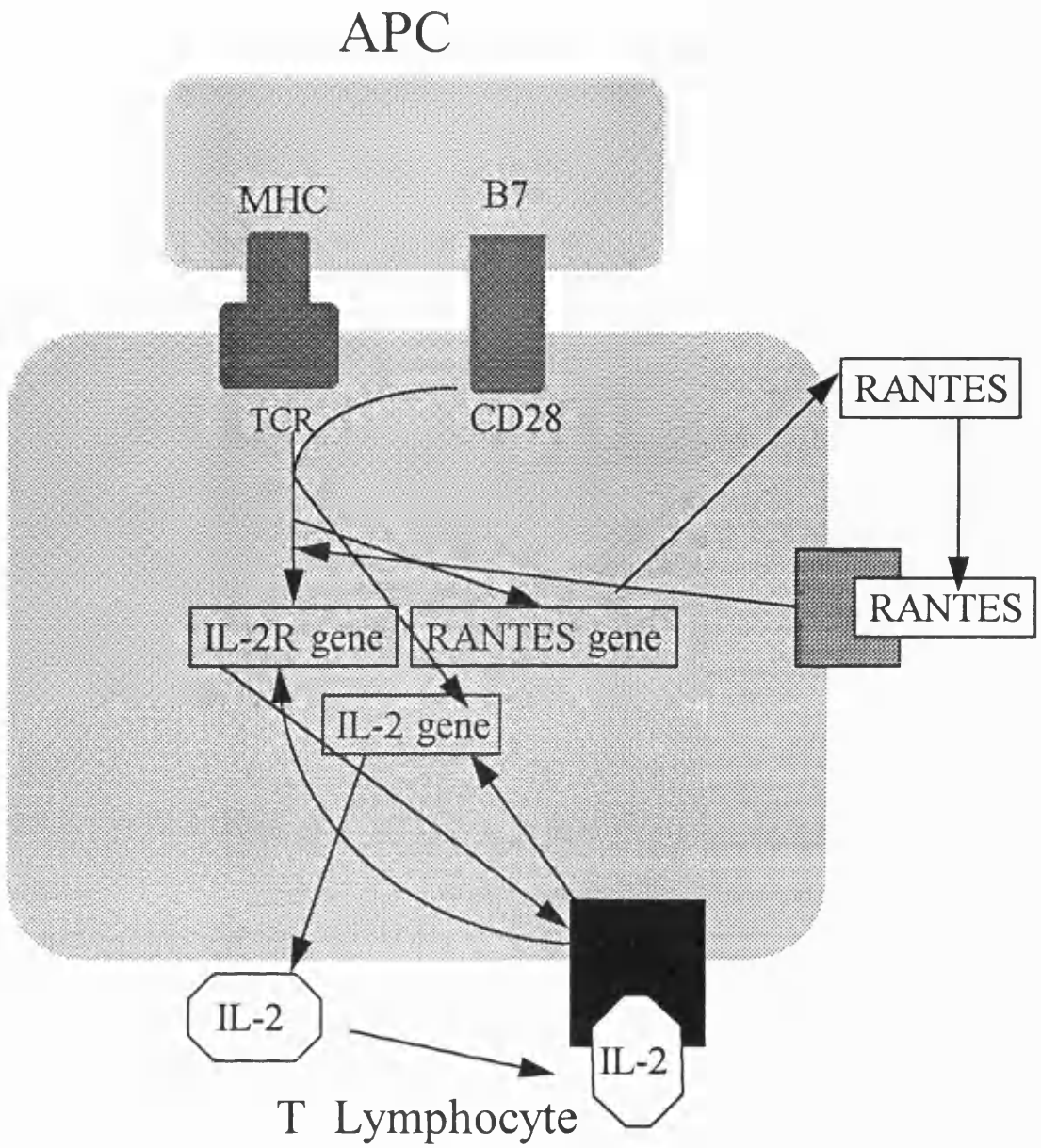


Fig. 4.2 Model of the Role of RANTES in Costimulation of T Lymphocytes

Presentation of an antigen by a APC to a T lymphocyte in the presence of the costimulatory signal not only increases IL-2 production and leads to IL-2R upregualtion, but causes an increase in RANTES production. RANTES released from the cells, could act in an autocrine manner on RANTES cell surface receptors and amplify the costimulatory response, resulting in increased proliferation and IL-2 receptor upregulation.

4.5 Conclusions

The conclusions that can be drawn from this study are several :

a) RANTES is a potent chemoattractant of primary T lymphocytes but has no effect on the migration of quiescent T lymphoblasts or the Jurkat T cell line suggesting that these transformed cells do not bind RANTES or more likely, have incomplete signalling pathways.

b) RANTES induced migration is preceded by RANTES induced shape change and actin polymerisation of T lymphocytes. These effects appear to be brought about through binding of the same RANTES receptor as a similar concentration of RANTES causes optimal induction of migration, polarisation and actin polymerisation.

c) The effects of RANTES on T lymphocytes do not seem to involve the classical phosphoinositide pathway as no elevation of $[Ca^{++}]_i$ was detected after addition of RANTES. This is also supported by the inability to detect PtdOh production or $PI(4,5)P_2$ breakdown after RANTES stimulation of T lymphocytes.

d) The PI 3-kinase signalling pathway is involved in RANTES signalling in T lymphocytes since PI 3-kinase activity is induced after treatment with RANTES and wortmannin, a PI 3-kinase inhibitor, inhibits RANTES induced migration, shape change, actin polymerisation and increase in PI 3-kinase activity. There is evidence to suggest that these effects of RANTES work through two different

PI 3-kinase enzymes as the IC_{50} for wortmannin inhibition of RANTES induced migration, shape change and actin polymerisation is much lower than the IC_{50} for wortmannin inhibition of RANTES induced increase in PI 3-kinase activity.

e) RANTES peptide is produced after costimulation of T lymphocytes. This result and the observation that proliferation induced by costimulation of T lymphocytes is partially inhibited in the presence of anti-RANTES indicates that RANTES has an important role in T lymphocyte proliferation. In addition the levels of proliferation seen after costimulation of T lymphocytes can be increased if the cells are costimulated in the presence of RANTES.

f) CHO-$B7^+$ stimulation of T lymphocytes only very slightly causes upregulation of the IL-2 receptor but if this treatment if repeated in the presence of RANTES, the upregulation is markedly increased. The upregulation of the IL-2 receptor detected after costimulation of the T lymphocytes is also upregulated in the presence of RANTES.

g) In contrast, production of IL-2 by T lymphocytes after costimulation is not increased if the costimulation is repeated in the presence of RANTES. Therefore, RANTES appears to upregulate the IL-2R without any significant effect on IL-2 production. The disparity in potentiating effects of RANTES in e), f), g) suggest that different RANTES signalling pathways are involved in the various physiological signals.

4.6 Implications

RANTES is a potent chemoattractant for T lymphocytes but the attraction of T lymphocytes to sites of infection is not its only role. RANTES potentiates the proliferation response of T lymphocytes to costimulation and so the production of RANTES peptide detected after costimulation of T lymphocytes will enhance the proliferative effects already observed. No other related C-C chemokines have been shown to have any effects on proliferation of T lymphocytes (Bacon *et al.* 1995) and so this gives RANTES an unique function in the C-C chemokine family.

The finding that RANTES stimulation of T lymphocytes at concentrations that induce optimal migration does not cause calcium mobilisation, suggests that calcium elevation and hence the classical phosphoinositide signalling pathway, is not linked to all C-C chemokine stimulation of T lymphocytes. However, the PI 3-kinase signalling pathway is involved in RANTES stimulation of T lymphocytes and so provides another target for therapeutic manipulation of the effects of chemokines.

A clearer understanding of the activation of leukocytes by RANTES and other chemokines may help to elucidate many complex immune responses and provide information about potential therapeutic treatment for diseases that RANTES is involved in, such as allergic inflammation and AIDS.

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